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<b>(54) Title:</b> INTERLEUKIN-2 BINDING PROTEIN FROM ARTHROPODS  <b>(57) Abstract</b>  The present invention provides arthropod interleukin-2 binding proteins, antibodies raised against such proteins and agonists and antagonists of such proteins. Methods of purifying such IL-2 binding proteins are also provided. The present invention further provides pharmaceutical compositions containing such proteins, antibodies, agonists or antagonists, as well as the use of such pharmaceutical compositions in protecting animals, including humans, from arthropod infestation and/or the transmission of infectious organisms by arthropods. The therapeutic compositions of the present invention can also be used to modulate the activity of the immune system of animals, including humans.		

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## INTERLEUKIN-2 BINDING PROTEIN FROM ARTHROPODS

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## FIELD OF THE INVENTION

The present invention relates to arthropod interleukin-2 binding proteins, antibodies raised against such proteins and agonists and antagonists of such proteins. The present invention also relates to pharmaceutical compositions containing such proteins, antibodies, agonists and antagonists, as well as the use of such pharmaceutical compositions in protecting animals, including humans, from arthropod infestation and/or the transmission of infectious organisms by arthropods. The therapeutic compositions of the present invention can also be used to modulate the activity of the immune system of animals, including humans.

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## BACKGROUND OF THE INVENTION

The black-legged or deer tick (*Ixodes scapularis*) is the main North American vector for Lyme Disease, now the most common vector-borne disease in the United States. Disease-causing parasites have evolved strategies to modulate host immune defenses, and tick immunosuppression of the host provides an environment favorable for transmission and establishment of tick-borne disease causing agents (such as *Borrelia burgdorferi* for Lyme Disease).

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Ticks suppress immune response pathways involved in both innate and acquired host resistance, by means of pharmacologically active agents present in the injected saliva, but this does not lead to the

death of the host, which would be counterproductive. These host defense modulatory phenomena (anti-hemostasis, anti-inflammatory and immunosuppressive) associated with tick feeding have been well documented in the literature for several species of ticks (*D. andersoni*, *I. ricinus*, *I. scapularis*), although none of the protein molecules mediating these effects have been identified or fully characterized.

The present inventors have identified a tick salivary protein complex that inhibits host T-cell proliferation (T-cell Inhibitor or Proliferation protein or pTIP) by binding and neutralizing mammalian IL-2. The lymphokine IL-2 is a glycoprotein (15-18 kDa) that is synthesized and secreted primarily by T helper lymphocytes that have been activated by stimulation with certain mitogens or by interaction of the T cell receptor complex with antigen/MHC complexes on the surfaces of antigen presenting cells. The response of T helper cells to activation is induction of the expression of IL-2 and IL-2 receptors (alpha, beta, gamma) with subsequent, clonal expansion of antigen-specific T-cells. The high affinity IL-2 receptor (IL2-R) is thought to be the alpha, beta, gamma heterotrimer subunits with an affinity for IL-2 of 10 pM. A soluble form of IL-2R alpha appears in the serum concomitant with its increased expression on cells. Its function is unclear, since it would be expected to be a poor inhibitor of IL-2 activity because of its low affinity for IL-2. Because of the central role of the IL-2/IL-2R system in mediation of the immune response, the identification and characterization of IL-2 binding proteins has important therapeutic implications. According to the present

invention, pTIP can be used as an immunosuppressive agent in the prevention of allograft rejection, providing an alternative to the currently available cyclosporin A, FK506 and rapamycin or as an anti-inflammatory agent for treating autoimmune-type diseases such as rheumatoid arthritis, diabetes, multiple sclerosis and psoriasis. In addition, pTIP can also be used in vaccines designed to reduce or eliminate vector/pathogen transmission.

#### SUMMARY OF THE INVENTION

The present invention relates to arthropod interleukin-2 binding proteins. In particular, the present invention provides the identification of a tick saliva protein complex that inhibits T cell proliferation (T cell Inhibitor of Proliferation or pTIP) by binding and neutralizing IL-2. Accordingly, the present invention is directed to isolated tick IL-2 binding proteins, antibodies raised against such proteins as well as agonists and antagonists of such proteins. The present invention is further directed to therapeutic compositions containing the instant proteins, antibodies and/or other agonists and antagonists. The present invention also contemplates the use of such therapeutic compositions in modulating the activity of the immune system of animals, including humans and in protecting animals, including humans, from tick infestation and/or tick-mediated transmission

of infectious organisms.

#### DESCRIPTION OF DRAWINGS

Figure 1 depicts the protein profiles of saliva and salivary gland.

5           Figure 2 depicts the protein profile of fractionated saliva.

Figure 3 depicts the assay of the effects of day 6 near replete saliva and saliva fractions on splenic T-cell proliferation.

10           Figure 4 depicts the saliva inhibition of PMA + ionomycin-induced splenic T-cell proliferation.

Figure 5 illustrates that the inhibitory effect of tick saliva on T-cell proliferation can be washed away.

15           Figure 6 Panel A depicts the inhibitory effects of saliva on the detection of IL-2 in the cell free capture ELISA assay. Panel B depicts the presence of similar IL-2 ELISA inhibitory activity in other tick species.

20           Figure 7 depicts the binding of both mouse, bio-rmIL-2, and human, bio-rhIL-2, IL-2 by plate-bound tick saliva.

Figure 8 depicts the Enzyme Interference Assay (EIA) of plate-bound saliva.

25           Figure 9 depicts the effects of tick saliva on human peripheral blood mononuclear cell (PBMC)/T cell proliferation.

30           Figure 10 depicts the silver stained SDS gels with the eluted proteins after affinity purification. The affinity purification support was coupled with rhIL-2 (Panel A) or no IL-2/no protein (Panel B). Panel C depicts the results of reiterative sequential

affinity purification. Panel D depicts the ability of an agarose support matrix but not polyacrylamide support to bind pTIP. Lanes as labeled in the figure represent: the supernatant following three sequential steps of affinity purification; primary (1°) affinity purification of pTIP; secondary (2°) affinity purification; tertiary (3°) affinity purification; molecular weight standards.

Figure 11 Panel A depicts silver stained SDS gel with saliva and protein size standards (kDa). Panel B and C depict Western blots of saliva probed with biotinylated recombinant murine IL-2 (bio-rmIL-2).

Figure 12 depicts the effects of various sugars on pTIP binding of IL-2 in a capture ELISA. NANA = N-acetyl neuraminic acid (sialic acid), Mucin - a salivary protein of mammals that is very highly sialylated, GlcNAc = N-acetyl glucosamine, GalNAc = N-acetyl galactosamine, Glc = glucose, Gal = galactose, Man = mannose, GalN = galactosamine, GlcN = glucosamine.

#### DETAILED DESCRIPTION OF THE INVENTION

Tick saliva has been known to produce anti-hemostatic, anti-inflammatory and immunosuppressive effects on the host that are believed to facilitate feeding as well as transmission of tick-borne pathogens. However, the molecules mediating these effects have not been completely characterized prior to the present invention. In accordance with the present invention, a tick saliva protein complex has been identified that inhibits T cell proliferation (T cell Inhibitor of Proliferation or pTIP) by binding and neutralizing IL-2.

One embodiment of the present invention is directed to the isolated tick *Ixodes scapularis* saliva protein complex, pTIP, which binds mammalian IL-2 and appears as two bands a non-reducing SDS gel with  
5 apparent molecular weights between 31 kD to 45 kD.

The term "protein" used herein refers to both the unmodified forms (precursors) and post-translationally modified forms of a protein.

Eukaryotic cells are well known for their ability to post-translationally modify proteins, so as to produce glycoproteins and lipoproteins, for example.  
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According to the present invention, pTIP has been found to be present in the 50-100 kD fraction of the tick saliva which is sequentially size-fractionated using ultrafiltration, a procedure well known in the  
15 art. pTIP migrates as two bands on a non-reducing SDS gel with apparent molecular weights between 31 kD to 45 kD. The discrepancy between the size determined by sequential size fractionation and that determined by an SDS-PAGE, is due to the fact that fractionation of  
20 molecules by ultrafiltration depends upon the shape, in addition to the mass, of the molecules.

The present invention has found that the pTIP-containing saliva fraction of tick *Ixodes scapularis*, inhibits T cell proliferation in *in vitro* assay systems. A variety of well-known systems can be employed to assay the effect of pTIP on T cell proliferation. These systems may vary in the source of T cells used for the assay (e.g., isolated mammalian  
25 splenocytes, or T cell lines maintained in culture), the means of stimulation (e.g., PMA (phorbol myristate acetate) plus ionomycin or anti-CD3 antibody), or the method of detection (e.g., the measurement of <sup>3</sup>H-  
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thymidine incorporation or a CTLL bioassay).

In accordance with the present invention, one assay system which can be used to determine the effect of pTIP on T cell proliferation is set forth as follows. Murine splenocytes are isolated and plated in the wells of microtiter plates at about  $1 \times 10^5$  per well. Whole saliva or a fraction of saliva is added to the wells, and the cells are then stimulated with anti-CD3 antibody. At about 24 hours post-stimulation, the cells are pulsed with 1  $\mu$ Ci of  $^3\text{H}$ -thymidine for about 20 hours and harvested afterwards. The amount of  $^3\text{H}$  incorporated into the cells can be determined by scintillation counts as a measure of T cell proliferation. Thus, a reduction in the amount of  $^3\text{H}$  incorporated is indicative of the inhibitory effect of the pTIP-containing saliva on T cell proliferation.

Another assay system which can be employed to determine the effect of pTIP on T cell proliferation involves the use of PMA plus ionomycin in stimulating T cells. The T cell proliferation can be measured similarly as described above. The combination of PMA and ionomycin is known in the art to be a powerful means of activating T cells. Thus, by using PMA plus ionomycin in lieu of anti-CD3, the potency of the inhibitory effects of pTIP can be illustrated.

According to the present invention, the effects of pTIP on the immune system of a host are not necessarily limited to the inhibition of T cell proliferation. As it is known in the art, IL-2 is the major autocrine growth factor of T cells. It is produced and secreted by T cells after they are stimulated by binding of antigen to the T cell antigen receptor (TCR). IL-2 also serves as a paracrine

activator of a wide variety of cells in the immune system. These cells include helper T cells (TH), cytotoxic T cells (CTL), B cells, natural killer cells (NK) and macrophages. The binding of IL-2 to the IL-2 receptor (IL-2R) results in the proliferation of the activated T cells and increased secretion of lymphokines as well as an increase in the surface expression of other growth factor membrane receptors, such as the transferrin and insulin receptors. IL-2 appears to play a very important role in the development of functional antigen-specific CTLs. After B cells are activated they also express high affinity IL-2R, making them responsive to IL-2 mediated signaling. Binding of IL-2 in conjunction with other signals plays a role in the differentiation of B cells into antibody secreting plasma cells. The activation of monocytes (macrophages) by IL-2 induces IL-1 secretion. IL-2 also promotes the proliferation of macrophage precursors and increases phagocytosis. Monocyte mediated cytotoxicity is also enhanced after IL-2 stimulation. NK cells proliferate in response to IL-2 stimulation. NK cells are also induced to produce IFN-gamma and become more phagocytic in response to IL-2. It is clear that an IL-2 binding and neutralizing protein can have effects on various effector cells of the immune system. Further, the effects of a decrease in available IL-2 would be expected to decrease or alter the activation state of T cells as well as the other cell types described above. These inhibitory effects would also be expected to be enhanced in cases where direct cell-cell interactions are also involved in the activation process, as is the case of TH-B cell interactions that result in antigen-specific B cell

activation and T cell interactions with antigen presenting cells (e.g., macrophage, dendritic cells and B cells. See, e.g., M.C. Howard, et al. (1993) *T-Cell Derived Cytokines and Their Receptors*, Third Edition, W.E. Paul ed., Raven Press, Ltd., New York, pp. 763-800; and K.A. Smith (1988) *Science* 240: 1169.

The present inventors have found that pTIP binds mammalian interleukin 2 (IL-2), particularly, murine and human IL-2. It is known that IL-2, synthesized and secreted primarily by T helper cells in response to stimulation of antigen or certain mitogens, is essential for clonal expansion of antigen-specific T cells. Thus, according to the present invention, pTIP in tick saliva can inhibit T cell proliferation by binding and neutralizing circulating IL-2 in host animals.

The binding of pTIP to IL-2 can be detected *in vitro* by a number of assays developed by the instant inventors. One of such assays, referred to hereinafter as a capture ELISA, detects the binding of pTIP to IL-2 by assaying the reduction of IL-2 that can be recognized and "captured" by antibodies specific for IL-2. More specifically, a sample containing pTIP (e.g., whole saliva or fractionated saliva from ticks) can be mixed with a sample containing a defined amount of IL-2. The mixture can be incubated for a period of time and at a temperature sufficient for the pTIP binding to IL-2 to be complete. Preferably, the incubation is maintained at ambient temperature for at least about half an hour, more preferably, for about 1-2 hours. The mixture is then added to ELISA plates pre-coated with a first anti-IL-2 antibody. It is routine in the art to coat a microtiter plate with

proteins such as antibodies. Afterwards, the plates are kept at, preferably, about 4°C, and for at least about 2 hours, preferably, about 12-16 hours.

Subsequently, the supernatants in the plates are washed away and a second anti-IL-2 antibody is added. The second anti-IL-2 antibody is labeled with a reporter enzyme, preferably HRP (horse radish peroxidase) or AP (alkaline phosphatase), which can give a detectable signal as the basis for measuring the IL-2 bound to pTIP on the microtiter plates. Those skilled in the art can readily modify the instant capture ELISA assay by, e.g., labeling the second anti-IL-2 antibody with, e.g., an isotope or a luminesceine in place of a reporter enzyme. In accordance with the present invention, both the primary and secondary anti-IL-2 antibodies can be monoclonal or polyclonal antibodies.

The present invention provides another assay that detects the pTIP binding of IL-2. In such assay, a microtiter plate is contacted with a pTIP-containing sample (e.g., whole saliva or fractionated saliva). The contact proceeds for a period of time and at a temperature that is sufficient for pTIP to be bound to the plate; preferably, for at least about 1 hour at about 4°C, and more preferably, for 12-16 hours at about 4°C. Afterwards, the plate is washed to remove the unbound material and then contacted with a solution containing a "blocking" reagent (e.g., BSA) that can reduce non-specific interactions in the steps that follow. The plate is then contacted with biotinylated IL-2 (bio-IL-2). Murine and human IL-2 that are commercially available or produced from a recombinant expression system can be employed according to the present invention. The biotinylation procedure is

well-known in the art and can be found in, e.g.,  
*Current Protocols in Immunology* (Coligan et al., John  
Wiley & Sons, New York). According to the present  
invention, murine IL-2 is preferred for use in  
5 biotinylation. Bio-IL-2 bound to the plate can be  
detected by addition of avidin conjugated with a  
reporter enzyme, e.g., HRP or AP. Various  
modifications of this assay can be made; for example,  
IL-2 can be radiolabeled rather than modified with  
10 biotin, or IL-2 is not labeled and is contacted with a  
detectably labeled anti-IL-2 antibody.

The present invention further provides that  
the specificity of the pTIP binding of IL-2 can be  
assessed by using an Enzyme Interference Assay (or  
15 EIA). More specifically, bio-IL-2 and IL-2 (non-  
biotinylated) can be added together to the pTIP-bound  
plates and compete for binding to pTIP. A reduction of  
bio-IL-2 bound to pTIP that is proportional to the  
concentration of non-biotinylated IL-2 is indicative of  
20 a specific IL-2 binding by pTIP.

Still another assay can be employed for  
detection of the pTIP binding of IL-2. A sample  
containing pTIP can be separated by SDS-PAGE and  
transferred to an appropriate membrane for subsequent  
25 Western Blot with biotinylated IL-2. The bio-IL-2  
molecules that are bound to the pTIP molecules on the  
membrane can be detected by contacting the membrane  
with streptavidin conjugated with a reporter molecule,  
such as HRP or AP, preferably in conjunction with a  
30 chemiluminescent detection system.

Further in accordance with the present  
invention, the pTIP binding of IL-2 can be detected by  
affinity chromatography. In this regime, mammalian IL-

2 molecules, preferably murine or human IL-2, are chemically crosslinked to a solid support chromatography material using routine chemical cross-linking procedures. Such procedures are described by, e.g., *Current Protocols in Immunology* (Coligan et al., John Wiley & Sons, New York). A preferred support material in accordance with the present invention is Hitrap-NHS support (Pharmacia). A sample containing pTIP is then brought into contact with such support to allow pTIP to bind to the IL-2 immobilized on the support. The support material is subsequently washed extensively to remove non-IL-2-binding molecules. The pTIP molecules bound to IL-2 are then eluted from the support using methods known in the art, such as solutions containing SDS. pTIP in the elutes can be detected by, e.g., running an SDS page followed by silver staining.

The present invention has also found that pTIP binds to both glycosylated and unglycosylated recombinant IL-2. By using recombinant IL-2 produced in insect cells such as *Trichoplusia ni* (capable of O-glycosylation), the pTIP binding of such IL-2 is inhibited by the presence of N-acetylated sugars, in particular, N-acetyl neuraminic acid (sialic acid), N-acetyl glucosamine and N-acetyl galactosamine.

According to the present invention, pTIP bears homology with one or more of the subunits of the mammalian IL-2 receptor (IL-2R). While the IL-2R is normally cell associated, soluble forms of this receptor have been identified.

IL-2R has been cloned and characterized and consists of three subunits,  $\alpha$ ,  $\beta$  and  $\gamma$ . The IL-2R  $\alpha$  and  $\beta$  subunits bind IL-2 with low and moderate

affinity, respectively, while the  $\gamma$  subunit does not bind IL-2. IL-2R  $\alpha/\beta$  and  $\beta/\gamma$  heterodimers bind IL-2 with intermediate affinity, while the  $\alpha/\beta/\gamma$  heterotrimeric complex binds IL-2 with the highest affinity. The IL-2R  $\beta$  and  $\gamma$  subunits are members of the cytokine receptor superfamily (CKR-SF) which is comprised of the IL-3R  $\alpha$  and  $\beta$  subunits, IL-4R, IL-5R  $\alpha$  and  $\beta$  subunits, IL-6R, IL-7R, IL-9R, IL-12R, G-CSFR, GM-CSFR, CNTFR, LIFR, EpoR, PRLR, GHR and gp130. Members of receptor superfamilies share putative domain structures. In addition, the IL-2R  $\alpha$  subunit, while not a member of the CKR-SF superfamily, has two domains that bear homology to the complement control protein superfamily (CCP-SF) which are involved in the control of the complement cascade. The IL-2R  $\gamma$  subunit is also a functional component of the IL-4R, IL-7R, IL-15R and may also be part of the IL-13R and IL-9R. IL-2R  $\beta$  and  $\gamma$  subunits are also part of IL-15R and the functional characteristics of IL-15 mimic to a significant degree those of IL-2. Accordingly, pTIP is contemplated to have binding affinities for any of the ligands of the receptors mentioned above in addition to IL-2, particularly IL-15, as well as for components of the complement system. See, R. Callard and A. Gearing (1994) *The Cytokine Facts Book*, Academic Press, New York.

According to the present invention, those skilled in art can make pTIP in a number of ways. PTIP can be isolated from tick saliva by, e.g., affinity purification based on IL-2 binding, sequential size fractionation, or combinations thereof. The starting material (i.e., ticks) required to obtain sufficient amount of pTIP may vary depending on the procedure

used. In general, the present invention provides that about 6 ml saliva can be collected from about 1000 ticks, and that about 20 µl saliva is considered to be the basal starting level for purification of pTIP.

5 Those skilled in the art can test and confirm the characteristics of the isolated protein according to the foregoing disclosure.

The binding of IL-2 can be attributed to one or more parts of pTIP. By "part", it is meant a  
10 contiguous fragment of pTIP of at least about 2 or 3 amino acids. Those skilled in the art can determine the parts of pTIP that are responsible for IL-2 binding. For example, various peptide fragments of pTIP can be made chemically by peptidase, by peptide  
15 synthesizer, or by a recombinant cloning and expression system. The IL-2 binding ability of the peptide fragments can be tested using any of the assays described hereinabove. Accordingly, the IL-2 binding parts of pTIP are also within the scope of the present  
20 invention.

The pTIP homologous proteins from tick species other than *Ixodes scapularis* can be similarly isolated, and thus, are also contemplated by the present invention. The present inventors have  
25 illustrated that pTIP activity is present in the saliva of the Ixodid ticks *Amblyomma americanum* and *Ixodes pacificus*, as saliva from these ticks also inhibited the IL-2 ELISA (Figure 6, Panel B).

"pTIP homologous proteins" as used herein  
30 refer to proteins that are homologous to pTIP with an amino acid similarity of at least about 40%, preferably, 60%, more preferably, 75%. The degree of homology as used herein is the similarity index

calculated using the Lipman-Pearson Protein Alignment program with the following choice of parameters: Ktuple = 2, Gap Penalty = 4, and Gap Length Penalty = 12.

The present invention contemplates pTIP  
5 homologs from ticks of the Ixodidae family, including species of the genera *Ixodes*, *Amblyomma*, *Aponomma*, *Haemaphysalis*, *Hyalomma*, *Dermacentor*, *Cosmiomma*, *Nosomma*, *Rhipicephalus*, *Anomalohimalaya*, *Rhipicentor*, *Boophilus*, and *Margaropus*. In particular, the species  
10 that are known vectors of pathogens and/or pests to domestic animals are contemplated by the present invention, including *Ixodes ricinus*, *Ixodes pacificus*, *Ixodes holocyclus*, *Amblyomma americanum*, *Amblyomma hebraeum*, *Amblyomma variegatum*, *Dermacentor andersoni*,  
15 *Dermacentor reticulatus*, *Rhipicephalus appendiculatus*, *Rhipicephalus sanguineus*, *Boophilus microplus*, and *Boophilus annulatus*. In addition to the ticks of the Ixodidae family, pTIP homologs may also be found in ticks of the Nuttalliellidae and Argasidae families, as  
20 well as other blood-feeding arthropods such as mosquitos, biting flies, fleas and mites.

Another embodiment of the present invention is directed to antibodies raised against pTIP. Such antibodies can be generated by using a full-length pTIP  
25 protein or portions thereof as an immunogen. For the purpose of raising antibodies, "a portion of pTIP" refers to a fragment of at least 8 or 9 contiguous amino acids of pTIP. Small pTIP fragments can be chemically synthesized given the amino acid sequence of  
30 pTIP.

Antibodies can be generated by injecting an effective amount of pTIP or portions thereof into a suitable animal, alone or in combination with an

adjuvant. Such animals can include rabbit, chicken, rat, mouse, goat, horse and the like. The present invention contemplates both polyclonal antibodies and monoclonal antibodies. The procedure for making polyclonal antibodies is well known in the art and can be found in, e.g., Harlow, E. and Lane, D., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, 1988. Monoclonal antibodies directed against pTIP can be produced by generating hybridoma cell lines expressing and secreting monoclonal antibodies. The procedure for producing monoclonal antibodies is also well known in the art and can be found in detail in, e.g., Harlow, E. and Lane, D. (1988). Additionally, it is within the scope of this invention to include secondary antibodies directed against anti-pTIP antibodies.

A further aspect of the present invention is directed to methods for purifying pTIP.

One method of the present invention for purifying pTIP employs the binding affinity of pTIP for IL-2. According to this method, an affinity purification matrix is crosslinked with IL-2. A variety of affinity matrices suitable for chemical cross-linking can be employed, such as activated sepharose or agarose supports (Pharmacia, Piscataway, NJ), or activated affi-gel supports (Bio-Rad, Hercules, CA). Further, activated support matrices with or without spacer arms, as well as supports linked to magnetic particles, are all suited for cross-linking. A preferred matrix material for cross-linking is Hitrap-NHS support (Pharmacia). Mammalian IL-2 molecules, preferably murine or human IL-2, are crosslinked to the matrix using routine chemical cross-linking procedures. Such procedures are described in,

e.g., *Current Protocols in Molecular Biology* (Ausubel et al., John Wiley & Sons, New York). A source material containing pTIP is secured, such as whole tick saliva, fractionated saliva, salivary gland lysate or recombinantly produced pTIP. Such sample can be brought into contact with the matrix support in tubes for small scale purification, or in chromatographic columns for large scale purification. The contact is generally carried out in a solution that favors the binding of pTIP to the IL-2 immobilized on the matrix. Preferably, such CBB solution containing 50 mM Tris pH7.5/0.5 mM NaCl. Afterwards, the matrix is washed extensively to remove non-IL-2-binding molecules. The pTIP molecules bound to IL-2 can then be eluted from the support using methods known in the art, such as solutions containing SDS at about 2% (w/v). pTIP in the elutes can be detected by running an SDS page followed by silver staining, or by a functional IL-2 binding assay described hereinabove.

Those skilled in the art can readily adjust the amount of IL-2 and the amount of matrix used for cross-linking. As a general rule, the present invention provides the following stoichiometry: 5  $\mu$ l Bed volume of matrix can be coupled with about 5-15  $\mu$ g of IL-2 for purifying pTIP from about 20  $\mu$ l of tick saliva. The purification efficiency can be further improved by collecting the supernatant or the efferent fraction off the affinity matrix for binding to, and purification by, an additional aliquot of affinity matrix. The same effects can be accomplished by the immobilization of IL-2 at a higher concentration on the matrix.

Another method of the present invention for

purifying pTIP, also based on the binding affinity of pTIP for IL-2, involves the use of biotinylated IL-2. According to such method, a sample containing pTIP is mixed with biotinylated IL-2 in a solution that permits the pTIP-IL2 binding. The solution is then mixed with a solid material coupled with avidin, preferably, monomeric avidin. The solid material can be agarose, sepharose or other matrices, in any form such as beads or column. PTIP, bound to bio-IL-2, is retained by such avidin coupled support, and other non-IL-2 binding materials can be easily removed. pTIP can then be collected by disrupting the pTIP-bio-IL2 interaction with, e.g., solutions containing SDS.

Another method of the present invention for purifying pTIP is based on the ability of agarose-based affinity support matrices to bind pTIP with a relatively low efficiency.

Another method of the present invention for purifying pTIP employs matrices coupled with N-acetylated sugars. N-acetyl neuraminic acid (sialic acid), N-acetyl glucosamine and N-acetyl galactosamine are preferred sugars for such purification. Such N-acetylated sugars can be coupled, i.e., cross-linked, to any matrices described hereinabove suitable for cross-linking. Matrices coupled with N-acetylated sugars that are commercially available can also be employed.

In another embodiment, the present invention provides a method of identifying an agonist/antagonist of pTIP using the capture ELISA assay of the present invention.

The present invention provides an assay system, cell-free capture ELISA, that detects pTIP

binding to IL-2. The present invention also provides that N-acetylated sugars, such as N-acetyl neuraminic acid (sialic acid), Mucin, N-acetyl glucosamine, or N-acetyl galactosamine, can inhibit such binding in the capture ELISA assay. Accordingly, the capture ELISA assay as described hereinabove can be used for identifying antagonists or agonists of pTIP from, e.g., pools of molecules manufactured by chemical combinatorics, or recombinantly produced parts of pTIP.

In another embodiment, the present invention contemplates pharmaceutical compositions containing, for example, the isolated pTIP protein or parts thereof, an anti-pTIP antibody, or combinations thereof.

As pTIP has been identified as a tick saliva protein complex by the present invention, isolated pTIP or parts thereof can be used as a vaccine in generating immunity in host animals against tick feeding.

According to the present invention, host animals can also be passively immunized with an anti-pTIP antibody.

It is known in the art that resistance to tick feeding can in some cases result in decreased transmission of diseases carried by ticks.

Accordingly, the isolated pTIP protein(s) or parts thereof, or an anti-pTIP antibody may be useful in reducing both tick feeding and transmission of pathogens.

In addition to the use as vaccines, the isolated pTIP protein(s) or parts thereof may also be used as an immunoregulatory reagent. For example, the isolated pTIP protein(s) or parts thereof when

administered to a host animal, are contemplated to inhibit the function of IL-2 thereby regulating the immune responses in such host animal including responses mediated by T cells, B cells or any other cells of the immune system, the functions of which are affected by IL-2. In addition, the isolated pTIP proteins can be used as a reagent to bind and neutralize IL-2 in any *in vitro* systems.

The pharmaceutical compositions of the present invention can include additional components, such as adjuvants, cytokines, chemokines and any other substances that may be appropriate.

The pharmaceutical compositions of the present invention can also include a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers which can be employed include, but are not limited to, any and all solvents, including water, lipids, dispersion media, culture from cell media, isotonic agents and the like that are non-toxic to the host. Except insofar as any conventional media or agent is incompatible with the pTIP reagent of the present invention, use of such conventional media or agent in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Another embodiment of the present invention provides a method of inhibiting tick feeding and/or transmission of pathogens on an animal by administering to such animal, a therapeutically effective amount of isolated pTIP or part thereof, or an antibody specific for pTIP or part thereof, alone or together with a pharmaceutical carrier. Such administration functions to generate immunity, either actively or passively,

against ticks. Immunity against the tick may result in decreased transmission of, or decreased incidence of infection with, tick-carried pathogens, such as pathogens causing Lyme disease, Ehrlichiosis and Babesiosis.

An "animal" as used herein refers to any mammal, including human, a domesticated animal or a livestock animal such as dog, cow, sheep, mouse, rabbit, pig, monkey, chicken, horse and the like; preferably, the animal is a human subject.

By "inhibiting" is meant preventing the occurrence, alleviate the severity or eliminate the syndrome of a disease condition.

Another embodiment of the present invention provides a method of regulating an immune response in an animal in need thereof by administering pTIP to such animal.

The present invention provides that pTIP binds and neutralize IL-2 thereby interfering the function of IL-2 on cells of the immune system. Thus, an immune response in an animal can be regulated, e.g., suppressed, by administering an effective amount of pTIP to such animal. This method of the present invention is particularly useful to animals having an immune disorder such as an autoimmune disorder (e.g., rheumatoid arthritis, diabetes, multiple sclerosis and psoriasis), or animals having received transplanted tissues. Such method of the present invention can also be used to regulate an immune response in an animal against another protein. For this purpose, pTIP is simply administered together with such other protein to such animal.

pTIP can be administered to the animal with

other materials that are appropriate, such as lymphokines, adjuvants, or a pharmaceutical acceptable carrier. Administration can be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, or transfusion. Preferably, the administration is carried out by injection, including subcutaneous (s.c.), intraperitoneal (i.p.), intra-arterial (i.a.) or intravenous (i.v.) injection.

The amount of a pTIP reagent required to be therapeutically effective for inhibiting tick feeding and/or transmission of pathogens on an animal, or for regulating an immune response, can be determined according to the age and the condition of the subject. Multiple administrations of the therapeutic compositions may be required, which can be determined by a physician.

According to the present invention, pTIP can also be used as a diagnostic antigen in Western blots to determine if an animal, preferably a human, has been exposed to tick feeding. In this application, the serum from said patient is used to probe immobilized pTIP, preferably recombinantly produced pTIP. pTIP can be immobilized either on microtiter plates, a membrane such as nitrocellulose, or other suitable solid supports commonly used in this type of analysis. The presence of antibodies to pTIP in the serum of the patient would indicate exposure to tick feeding. Relevant teaching can be found in, e.g., M.L. Sanders et al. (1998) *Am. J. Trop. Med. Hyg.* 59(2): 279-285.

The following Examples are intended to illustrate, but not to limit the invention. While such Examples are typical of those that might be used, other procedures known to those skilled in the art may

alternatively be utilized.

The teachings of the publications cited throughout the present specification are herein incorporated by reference.

**EXAMPLE 1****Protein Profiles of Saliva  
and Salivary Gland Extract**

5               Female Ixodes scapularis were collected in  
the field at Bridgeport, Connecticut. Ticks were  
maintained and fed essentially according to D.E.  
Sonenshine (1993) *The Biology of Ticks*, vol.2, Appendix  
pp. 372-412, Oxford University Press, New York.

10              Salivary glands and saliva were collected from ticks  
fed on rabbits. Salivary gland lysate was prepared by  
ultrasonic disruption of salivary glands in phosphate  
buffered saline, followed by centrifugation at 16,000 x  
g which cleared insoluble gland debris from the lysate.  
15              Thirty microliters of saliva and salivary gland lysate  
containing 10 µg protein were loaded onto a Tris-  
Glycine SDS-PAGE gel (15% total acrylamide, 0.5% bis-  
acrylamide, 10% glycerol separating gel with a 3.9%  
total acrylamide, 4.8% bis-acrylamide, 10% glycerol  
20              stacking gel) (Laemmli, *Nature* 227: 680, 1970). The  
protein profiles were visualized by staining the SDS  
gel with sypro orange (BioRad, Hercules, CA).

              Figure 1 illustrates the relatively simple  
protein profile of the saliva compared to that of a  
25              whole salivary gland lysate. The size range of the T-  
cell Inhibitor of Proliferation proteins(s), pTIP, is  
indicated by the bracket on the right. Saliva proteins  
in this size range were separated into three distinct  
bands using this gel formulation. A better separation  
30              of proteins in this size range was achieved with  
NuPAGE-SDS-MOPS gels (NOVEX, San Diego, CA), which made  
more bands apparent. At least eight saliva proteins in  
this size range were observed in two-dimensional gels.

EXAMPLE 2**Splenic T-cell Proliferation Assay With  
Day 6 Near Replete Saliva and Saliva Fractions**

5           The effects of different saliva fractions on  
T-cell proliferation were tested as follows. Sixty  
microliters of saliva from female *Ixodes scapularis* was  
subject to sequential size fractionation using  
ultrafiltration (Microcon filters, Amicon,  
10       Inc./Millipore). Filtration fractions were collected  
in 15 µl of 1 x PBS pH 7.4.

Splenocytes from a female C57Bl/6J mouse were  
plated in microtiterplates at  $1 \times 10^5$  cells per well.  
Whole saliva or various fractions of saliva were added  
15       to the assay at a final dilution of 1:50. The cells  
were stimulated with anti-CD3 antibody (produced by  
cell line 145-2C11, ATCC # CRL-1975; Leo et al., *Proc.*  
*Natl. Acad. Sci. USA* 84: 1374-1378, 1987) immediately  
after addition of saliva. At 24 hours post-  
20       stimulation, the wells were pulsed with 1 µCi of  $^3\text{H}$ -  
thymidine for 20 hours, harvested and scintillation  
counted to determine the amount of  $^3\text{H}$  incorporated.  
Samples were run in triplicate, and the results were  
shown in Figure 3 with standard deviations.  
25       Percentages within the bars indicate the percent  
inhibition of proliferation relative to the positive  
control sample which contained no saliva.

As illustrated in Figure 3, the >50 kDa (<  
100 kDa) fraction of tick saliva inhibited the anti-CD3  
30       mediated stimulation of T-cell proliferation, and thus  
contained pTIP.

Five microliters of each collected fraction  
was run on an SDS-PAGE gel. The "whole saliva" lane  
contained 10 µl of unfractionated saliva from the same

pool of saliva used for fractionation. The gel was silver stained.

The gel image (Figure 2) illustrates the profile of proteins in the different ultrafiltrated fractions. As shown in Figure 2, the majority of proteins collected in the > 50 kDa fraction appeared to be less than 50 kDa on a reducing and denaturing gel. For comparison with Figure 1, the same area of the gel was bracketed at the right. As can be noted, the proteins bracketed on the SDS-PAGE were found in the > 50 kDa fraction. The arrow indicates the fraction where pTIP activity was found in proliferation assays (see Figure 3).

This indicates that the functional pTIP protein may be a multimer. However, ultrafiltration cut-offs are only approximate and depend upon the shape as well as the mass of molecules. Thus, the pTIP protein of about 36 kDa may fractionate in the >50 kDa fraction, even if pTIP is a monomer.

The effectiveness of pTIP in tick saliva on human T cells was demonstrated by the ability of tick saliva to inhibit the proliferation of human PBMC. Saliva at 1:100 and 1:200 final dilution was added to  $2 \times 10^5$  PBMC per well from 5 different human donors and stimulated with 2  $\mu\text{g/ml}$  PHA. At 24h post-stimulation the cells were pulsed for 18 h with  $[3\text{H}]\text{TdR}$ . Cultures were interrupted by harvesting and the proliferative response was measured by scintillation counting. Results are presented as the mean of triplicate samples. Proliferation after treatment with both saliva dilutions is significantly different from the (+) samples that were not treated with saliva, and from each other ( $p < 0.05$ ).

EXAMPLE 3**Saliva Inhibited PMA + Ionomycin-Induced  
Proliferation of Splenic T-cells**

5                   In order to determine the potency of the  
inhibitor of T-cell proliferation, PMA plus ionomycin,  
one of the most powerful means of activating T-cells,  
was used in the following experiment.

                  Splenocytes were obtained from a female  
10 C57Bl/6J mouse. Cells were plated at  $1 \times 10^5$  per well,  
and salivary gland extract was added at a final  
dilution of 1:50. Cells were stimulated with 1  $\mu\text{g/ml}$   
of PMA and 14  $\mu\text{M}$  ionomycin in a 100  $\mu\text{l}$  volume. Cells  
were pulsed with 1  $\mu\text{Ci}$  per well  $^3\text{H}$ -thymidine at the  
15 indicated time points and harvested at 24 hours after  
the pulse followed by scintillation counting to  
determine  $^3\text{H}$  incorporation. Samples were run in  
triplicate.

                  As shown in Figure 4, tick saliva inhibited  
20 PMA-ionomycin-mediated T-cell proliferation by about  
50% at each time point.

EXAMPLE 4**The Inhibitory Effect of Tick Saliva on  
T-cell Proliferation Was Washed Away**

5                   Splenocytes for the assay were obtained from  
a female C57Bl/6J mouse and treated in a batch format  
at  $5 \times 10^6$  cells in a 1.0 ml volume with saliva at a  
1:250 dilution. After pre-incubation with saliva for 2  
and 4 hours, the cells were washed twice before plating  
10                   and stimulation with anti-CD3. The cells were pulsed  
with  $^3\text{H}$ -thymidine at 22.5 hours post-stimulation and  
harvested after 18.5 hours, followed by scintillation  
counting to determine the  $^3\text{H}$  incorporation. Samples  
were run in triplicate.

15                   As indicated in Figure 5, the inhibitory  
effect of tick saliva on T-cell proliferation was  
"washed away." This indicates that pTIP acted  
indirectly on T-cells to mediate the inhibitory effect.

**EXAMPLE 5****IL-2 Binding Assays*****Saliva Inhibited IL-2 Detection in a Cell-free Capture ELISA***

5                   Saliva at an amount as indicated (Figure 6)  
was pre-incubated with IL-2 in 5% FBS-DMEM in  
polypropylene plates for 4 hours. The mixture was  
subsequently aliquoted to the ELISA plates containing  
primary IL-2 capture antibody (Pharmingen #18161D).  
10   Following overnight incubation at 4° C, the  
supernatants were washed away, and the secondary  
antibody conjugated with HRP (Pharmingen #18172D) was  
added. Both the primary and the secondary antibodies  
were obtained from Pharmingen (San Diego, CA). The  
15   ELISA was run essentially according to the instructions  
provided by the antibody supplier. Signals were  
detected using standard HRP developing reagents  
obtained from KPL (Gaithersburg, MD).

20                   Figure 6 shows that the incubation of native  
mIL-2 from conditioned culture medium with saliva  
resulted in the elimination of detectable IL-2 in the  
capture ELISA. Percent inhibition depended upon the  
concentrations of both saliva and IL-2 in the assay.  
This result indicates that saliva bound to IL-2 and  
25   blocked fully or partially one or both of the epitopes  
recognized by the monoclonal antibodies in the capture  
ELISA, resulting in the reduction of IL-2 that could be  
detected in the capture ELISA assay.

***Plate-bound Saliva Bound Recombinant Murine and Human  
IL-2***

30                   Recombinant murine IL-2 (rmIL-2) was obtained

from Genzyme (Cambridge, MA). Biotinylation of rmIL-2 was performed by following the standard procedure of reacting NHS-biotin with proteins in solution. See, e.g., Coligan et al. *Current Protocols in Immunology*, John Wiley & Sons Inc., New York, New York (1994).

Saliva from partially fed ticks was plated in carbonate buffer pH 9.6 on a standard ELISA plate overnight at 4°C. The plate was washed and then blocked with 3% BSA/0.1% tween-20/1X PBS pH 7.4. Following a wash, the plate was incubated with biotinylated rmIL-2 in 5% FBS/1X PBS for 2 hours at room temperature. The plate was washed and then incubated with avidin-HRP for 30 minutes. After a final set of washes, the plate was developed and stopped before OD at 450 nm was measured. Samples were run in triplicate. The value of a set of blank samples was subtracted from the data before graphing. The blank consisted of wells without saliva plated, treated as described above (the average blank value was 0.191, n = 5).

As indicated in Figure 7, the titration of the signal corresponded to the amount of saliva plated, thus demonstrating directly that saliva bound IL-2. Control samples did not bind biotinylated recombinant mouse IFN-gamma (bio-IFN-gamma), indicating that the IL-2 binding is specific.

#### ***Enzyme Interference Assay (EIA) of Plate Bound Saliva***

The specificity of the plate-bound saliva for IL-2 was measured using an EIA. The ligand in the assay was bio-rmIL-2, and competitor was rmIL-2. RmIL-2 was obtained from PharMingen (Product #19002BV). Biotinylation of a portion of the rmIL-2 was performed.

Wells of ELISA plate were coated with 1.5  $\mu$ l saliva in carbonate buffer pH 9.6 overnight at 4°C. Wells were washed and blocked with 3% BSA/0.1% tween-20/1X PBS pH 7.4 for two hours. The substrate, biotinylated rmIL-2 at approximately 13 pg per sample, was mixed with various concentrations of competitor, rmIL-2, in 5% FBS/1X PBS pH 7.4, and was then transferred to the ELISA plate for a 2-hour incubation at room temperature. The plate was washed and then incubated with avidin-HRP for 30 minutes. After a final set of washes, the plate was developed and stopped before OD at 450 nm was measured. Samples were run in duplicate, and the average OD was compared with the OD of the control sample in which no competitor was added.

As it was difficult to accurately determine the recovery of biotinylated rmIL-2, the fold of competitor values given on the X axis of Figure 8 is approximate. As indicated in Figure 8, the competition of the signal was complete at 8-fold and above excess competitor. Further, the signal titrated out with increasing competitor, thus the interaction of saliva with IL-2 was specific. In addition, all preparations of commercially obtained recombinant IL-2 contained various levels of carrier protein (usually BSA). When bio-rIL-2 was prepared, these proteins were also biotinylated, in some cases making bio-BSA the major protein species present in the reagent (depending on the manufacturer of rmIL-2). This experiment was carried out on plates blocked with BSA, and incubations were carried out in 5% FBS (which contained a large amount of BSA estimated at 10,000 fold excess versus IL-2 present). Thus, the ability to titrate out the

signal with competitors at pg levels indicated that the assay was specific for IL-2 binding of saliva.

**EXAMPLE 6****N-Acetylated sugars inhibited pTIP**

5 To investigate whether the glycosylation  
state of IL-2 affected the ability of saliva to bind  
IL-2, the following sugars at a 5 mM concentration were  
included in the IL-2 capture ELISA pTIP assay system:  
N-acetyl neuraminic acid (sialic acid), Mucin (a highly  
sialylated mammalian salivary ), N-acetyl glucosamine  
10 (GlcNAc), N-acetyl galactosamine (GalNAc), glucose  
(Glc), galactose (Gal), mannose, galactosamine (GalN)  
and glucosamine (GlcN). Percent inhibition of the pTIP  
activity was graphed and shown in Figure 12.

15 The N-acetylated sugars, NANA, GlcNAc, GalNAc  
and Mucin, inhibited the binding of saliva to IL-2 in  
the capture ELISA, whereas the other sugars had no  
appreciable effect on pTIP. Interestingly, NANA is  
known to be at the end of the sugar chain in native IL-  
2.

20

EXAMPLE 7

## Affinity Purification of pTIP

Recombinant human IL-2 (rhIL-2) was obtained from PeproTech (Rocky Hill, NJ) and was used in affinity purification of pTIP from 20 µl of partially fed female tick saliva. The affinity support was prepared by binding of rhIL-2 at approximately 1 µg IL-2/µl HiTrap-NHS support (Pharmacia) in polypropylene tubes following the protocol provided by the manufacturer. A 5 µl bed volume of this affinity support equilibrated in 1X Column Binding Buffer (1X CBB = 50 mM Tris pH7.5/0.5 mM NaCl) was incubated with 20 µl of saliva in a final volume of 25 µl 1X CBB. Incubation was kept for 30 min at room temperature, with gentle resuspension of the affinity support every few minutes. The support was then spun down at 3,000 x g and the supernatant was removed. This was followed by a series of 25 µl washes in the following order: twice in 10 mM Tris-Cl pH 8/140 mM NaCl, once in 50 mM Tris-Cl/500 mM NaCl pH 8, and once in 50 mM Tris-Cl/500 mM pH 9. Proteins bound to the support were eluted from the support with 25 µl of 2% SDS-PAGE sample buffer (NOVEX) at 70°C for 10 min. Samples were run on non-reducing 10% NuPAGE-SDS-MOPS gels (NOVEX, San Diego, CA) followed by staining with silver stain plus (BioRad, Hercules, CA). Standards were reduced. The gel was purposely overstained in order to achieve maximum exposure of all protein bands. Two bands of approximately 33 kDa and 36 kD were observed in the sample buffer elution lane (Panel A).

The experiment described in Panel A was repeated except that the HiTrap support used in the purification was not bound with rhIL-2 or any other

protein. Instead the support was processed as above and quenched to block the NHS groups before it was used to bind saliva using the same procedure as above (Panel B). The 33 kD and 36 kD bands observed in Panel A were  
5 no longer present in the sample buffer elution (Panel B). This indicated that the purification of these bands was due to their specific affinity to rhIL-2, rather than non-specific interaction with the column support.

10 To determine whether the affinity purification was quantitative, 5 µl affinity support containing approximately 5 µg rhIL-2 (the same stoichiometry as in Panel A) was used to affinity  
15 purify 20 µl of saliva (Panel C). In order to increase the likelihood of specific interactions, 0.05% tween-20 was included in all column solutions. Twenty microliters of saliva was incubated with 5 µl bed volume aliquots of affinity support. The supernant containing the excess saliva proteins not bound to the  
20 support material (the primary affinity support) was added to a second aliquot of affinity support material (the secondary affinity support). Similarly, the supernant containing the excess saliva proteins not bound to the secondary support was added to a third  
25 aliquot of affinity support material. All three supports were washed 4 times with 10 mM Tris-Cl pH 8/140 mM NaCl/0.05% tween-20, and proteins were eluted from each affinity support using 2% SDS sample buffer without reducing agent for 10 min at 70°C. Samples  
30 containing the eluted proteins from each purification step were run on a non-reducing 10% NuPAGE-SDS-MOPS Gel, which was subsequently silver stained with the NOVEX silverXpress system. Molecular standards were

reduced. See Figure 10.

As can be seen from Panel C, the efficiency of the affinity purification was improved by using 2-3 fold more IL-2 coupled affinity matrix.

5 As indicated in Panel D, agarose support matrices have a limited affinity for pTIP in the absence of an immobilized ligand as evidenced by the partial elimination of pTIP activity after incubation with an agarose support. This is in contrast to  
10 polyacrylamide based supports which do not bind pTIP. Therefore, it is possible to use unmodified agarose supports to purify pTIP with low efficiency.

**EXAMPLE 8****Western Blots of Saliva Probed  
with Biotinylated rmIL-2**

5           Ten microliters of non-reduced saliva was  
separated on a 10% NuPAGE-SDS gel run in SDS-MOPS  
buffer (NOVEX). The gel was silver stained and shown  
in Panel A. The protein standards were reduced.

10           Ten microliters of saliva was separated on a  
10% NuPAGE-SDS gel in SDS-MOPS buffer (NOVEX), and  
transferred to nitrocellulose using standard  
tris/glycine/methanol buffer (Coligan et al. *Current*  
*Protocols in Immunology*, John Wiley & Sons Inc., New  
York, New York (1994)). The membrane was blocked with  
15   3% BSA in TTBS overnight and then probed with a 1:2,500  
dilution of biotinylated recombinant murine IL-2 for 2  
hours at room temperature. Bio-rmIL-2 was produced as  
described in Example 5. The blot was then incubated  
with streptavidin-HRP and detected using  
20   chemiluminescent developing reagents and exposure for 4  
min according to the manufacturer's instructions (ECL,  
Amersham, Arlington Heights, IL). The blot is shown in  
Panel B.

25           As shown in Panel B, several bands were  
evident in the blot probed with bio-rmIL-2. However,  
the preparation of rmIL-2 contained a high level of  
BSA, thus, it was possible that the bands represented  
proteins that were recognized by the non-specific  
proteins present in the preparation of bio-rmIL-2. The  
30   bands of 37.3 kDa and 40.5 kDa were also observed in  
Panel C of Figure 10. The bands at 37.3 kDa and 40.5  
kDa, as well as the high molecular weight bands, were  
also present in control blots probed with bio-BSA and  
thus, were most likely due to non-specific protein

interactions (these bands are denoted with "\*" in Figure 11). Together with the affinity purification experiments (Figure 10), the bands at 35.2 kDa and 32.6 kDa are believed to be pTIP. When the saliva sample was reduced prior to separation on SDS-PAGE, the band at 32.6 kDa disappeared while the band at 35.2 kDa intensified. This indicates that pTIP may exist as isoforms with different conformations due to the presence of a disulfide bond(s) or possibly some other types of modification.

Panel C illustrates a blot of a 10 $\mu$ l non-reduced saliva sample probed with bio-rmIL-2 (1:5000). The experiment was carried out in the same manner as in Panel B, except bio-rmIL-2 was prepared using rmIL-2 obtained from PeproTech, which contained a lower level of contaminating BSA.

Example 9**Saliva of Other Ixodid Ticks Contains pTIP**

5                   As described in Example 5, saliva from *Ixodes*  
*scapularis*, *Ixodes pacificus* and *Ambloomma americanum* in  
the amount indicated in Figure 6, Panel B was  
preincubated with IL-2. The preincubated samples were  
then transferred to ELISA plates coated with the  
10           primary IL-2 capture antibody and the assay was  
completed as in the Example 5 description of the cell-  
free IL-2 capture ELISA assay for pTIP. As seen from  
Figure 6, Panel B, the saliva of the Ixodid tick  
species *Ixodes pacificus* and *Ambloomma americanum*  
15           inhibited IL-2 detection in the capture ELISA. Thus,  
the saliva of these tick species contains pTIP  
activity.

## WE CLAIM:

1. An isolated tick saliva protein complex pTIP, wherein said pTIP is capable of binding mammalian IL-2 and appears on a non-reducing SDS-PAGE gel as two bands with apparent Mw between 31 kD and 45 kD.

2. An isolated arthropod protein complex, wherein said protein complex is substantially homologous to said pTIP of claim 1.

3. The isolated tick saliva protein complex of claim 2, wherein said arthropod is a member of the *Ixodidae* family.

4. An antibody specific for the tick saliva protein complex pTIP, wherein said pTIP is capable of binding a mammalian IL-2 and appears on a non-reducing SDS-PAGE gel as two bands with apparent Mw between 31 kD and 45 kD.

5. A pharmaceutical composition comprising an isolated tick saliva protein pTIP and a pharmaceutical carrier, wherein said protein pTIP is capable of binding a mammalian IL-2 and appears on a non-reducing SDS-PAGE gel as two bands with apparent Mw between 31 kD and 45 kD.

6. A pharmaceutical composition comprising an antibody and a pharmaceutical carrier, wherein said antibody is specific for the tick saliva protein complex pTIP and wherein said pTIP is capable of binding a mammalian IL-2 and appears on a non-reducing

SDS-PAGE gel as two bands with apparent Mw between 31 kD and 45 kD.

7. A method of purifying tick saliva protein pTIP, comprising:

- a. cross-linking IL-2 molecules to a matrix;
- b. contacting the matrix obtained from step (a) with a sample containing pTIP to allow the binding of pTIP to IL-2;
- c. washing the matrix to remove unbound molecules; and
- d. eluting the matrix thereby obtaining purified pTIP.

8. A method of purifying tick saliva protein pTIP, comprising:

- a. mixing a sample containing pTIP with biotinylated IL-2 in a solution thereby forming a biotinylated-IL-2-pTIP complex in said solution;
- b. contacting the solution of step (a) with a matrix coupled with avidin thereby allowing the binding of the complex formed in step (a) to said matrix;
- c. washing the matrix to remove unbound molecules; and
- d. eluting the matrix thereby obtaining purified pTIP.

9. A method of purifying tick saliva protein pTIP, comprising:

- a. obtaining a matrix cross-linked with an N-acetylated sugar;

b. contacting the matrix of step (a) with a sample containing pTIP thereby allowing the binding of pTIP to the matrix;

c. washing the matrix to remove unbound molecules; and

d. eluting the matrix thereby obtaining purified pTIP.

10. A method of purifying tick saliva protein pTIP, comprising:

a. obtaining a matrix of unmodified agarose beads;

b. contacting the matrix of step (a) with a sample containing pTIP thereby allowing the binding of pTIP to the matrix;

c. washing the matrix to remove unbound molecules; and

d. eluting the beads thereby obtaining purified pTIP.

11. A method of identifying an antagonist of pTIP, comprising:

a. obtaining a sample containing pTIP and a candidate molecule suspected to be a pTIP antagonist;

b. coating a first and a second microtiter plate with a first anti-IL-2 antibody;

c. contacting the first plate from step (b) with the sample containing pTIP from step (a) thereby allowing pTIP to bind IL-2 on the plate;

d. contacting the first plate from step (c) with a second anti-IL-2 antibody, wherein said second anti-IL-2 antibody is detectably labeled and recognizes pTIP bound on the plate;

e. determining the amount of the second anti-IL-2 antibody bound on the first plate;

f. mixing the sample containing pTIP with the candidate molecule suspected to be a pTIP

5 antagonist from step (a);

g. contacting the second plate from step (b) with the mixture formed in step (f) thereby allowing pTIP to bind IL-2 on the plate;

10 h. contacting the second plate from step (g) with a second anti-IL-2 antibody, wherein said second anti-IL-2 antibody is detectably labeled and recognizes pTIP bound on the plate;

i. determining the amount of the second anti-IL-2 antibody bound on the second plate; and

15 j. comparing the value obtained in step (i) with the value obtained in step (e), a smaller value in step (i) being an indicative that the candidate molecule is a pTIP antagonist.

20 12. A method of identifying an agonist of pTIP, comprising:

a. obtaining a sample containing pTIP and a candidate molecule suspected to be a pTIP agonist;

25 b. coating a first and a second microtiter plate with a first anti-IL-2 antibody;

c. contacting the first plate from step (b) with the sample containing pTIP from step (a) thereby allowing pTIP to bind IL-2 on the plate;

30 d. contacting the first plate from step (c) with a second anti-IL-2 antibody, wherein said second anti-IL-2 antibody is detectably labeled and recognizes pTIP bound on the plate;

e. determining the amount of the second

anti-IL-2 antibody bound on the first plate;

f. mixing the sample containing pTIP with the candidate molecule suspected to be a pTIP agonist from step (a);

5 g. contacting the second plate from step (b) with the mixture formed in step (f) thereby allowing pTIP to bind IL-2 on the plate;

h. contacting the second plate from step (g) with a second anti-IL-2 antibody, wherein said  
10 second anti-IL-2 antibody is detectably labeled and recognizes pTIP bound on the plate;

i. determining the amount of the second anti-IL-2 antibody bound on the second plate; and

j. comparing the value obtained in step (i)  
15 with the value obtained in step (e), a larger value in step (i) being an indicative that the candidate molecule is a pTIP agonist.

13. A method of regulating an immune  
20 response in an animal in need thereof, comprising administering a therapeutically effective amount of pTIP to said animal for a time and under conditions sufficient to regulate said immune response.

25 14. The method of claim 13, wherein said animal is human.

15. The method of claim 13, wherein said  
30 animal has an autoimmune disorder.

16. The method of claim 13, wherein said  
immune response is against transplanted tissues.

17. A method of inhibiting tick feeding in an animal, comprising administering to said animal an effective amount of isolated pTIP or an antibody specific for pTIP, for a time and under conditions sufficient to inhibit said tick feeding.

18. The method of claim 17, wherein said animal is human.

19. A method of preventing tick transmitted pathogenic infection in an animal, comprising administering to said animal a therapeutically effective amount of isolated pTIP or an antibody specific for pTIP, for a time and under conditions sufficient to prevent said tick transmitted pathogenic infection.

20. The method of claim 19, wherein said animal is human.

21. The method of claim 19, wherein said pathogenic infection is Lyme disease, Ehrlichiosis, or Babesiosis.

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FIG. 1

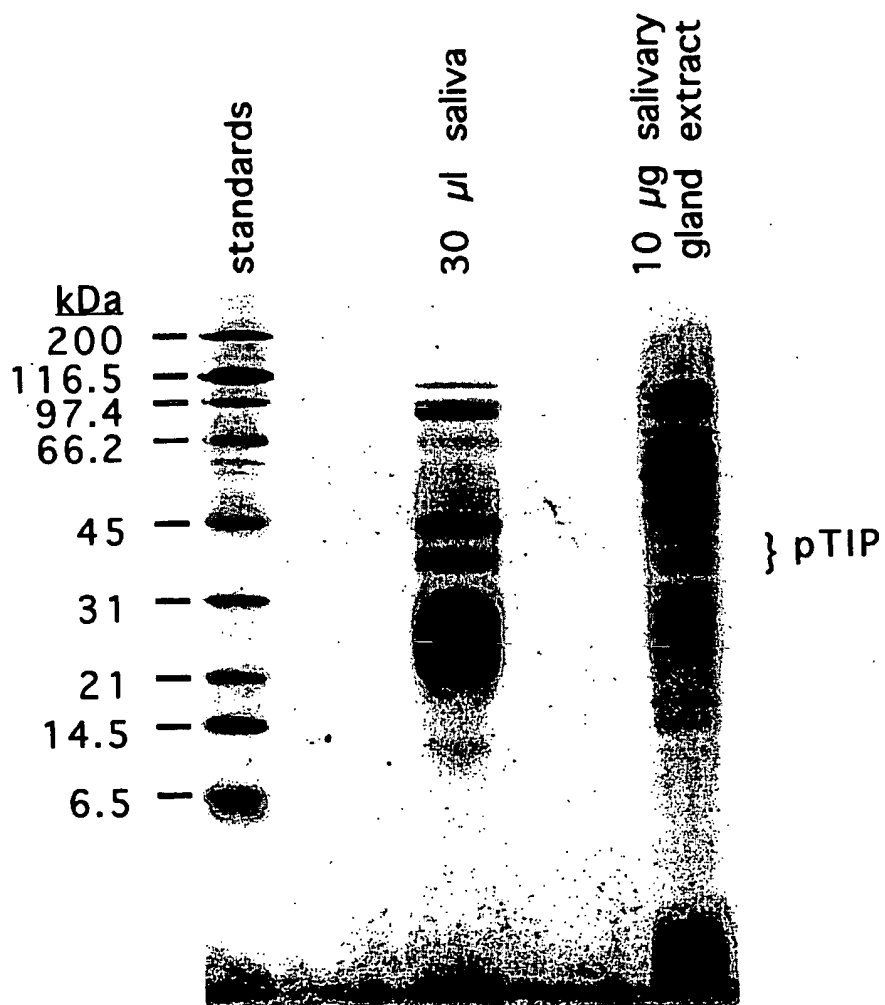


FIG. 2

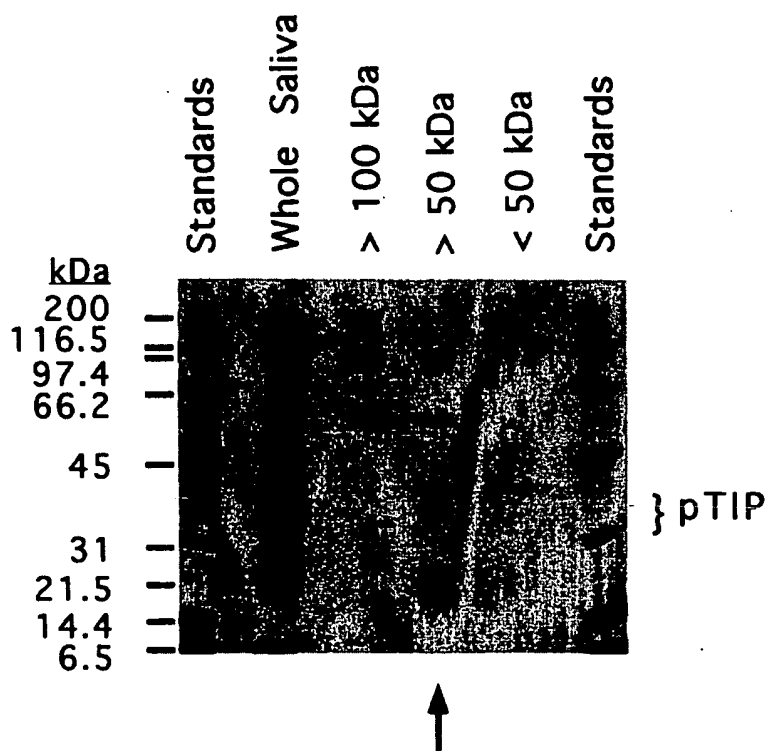
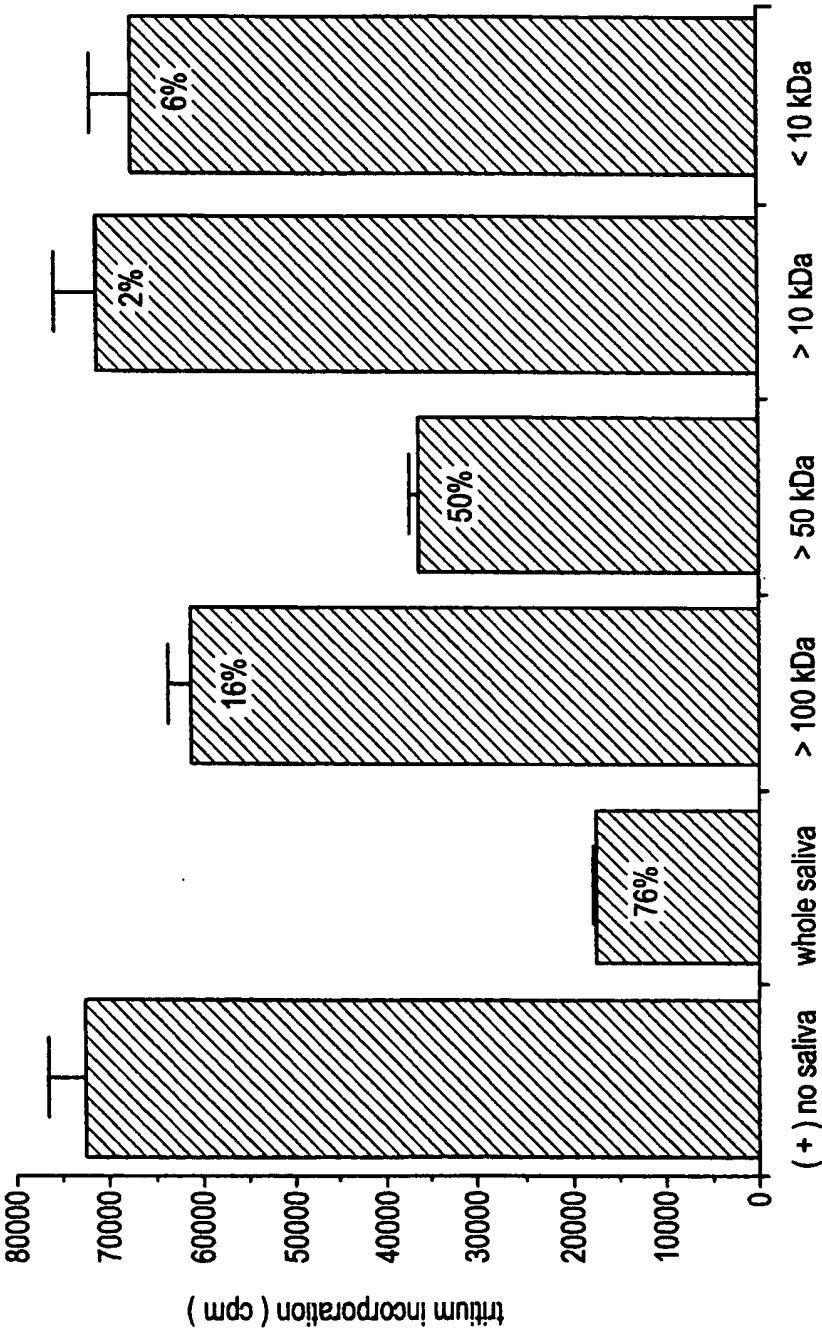
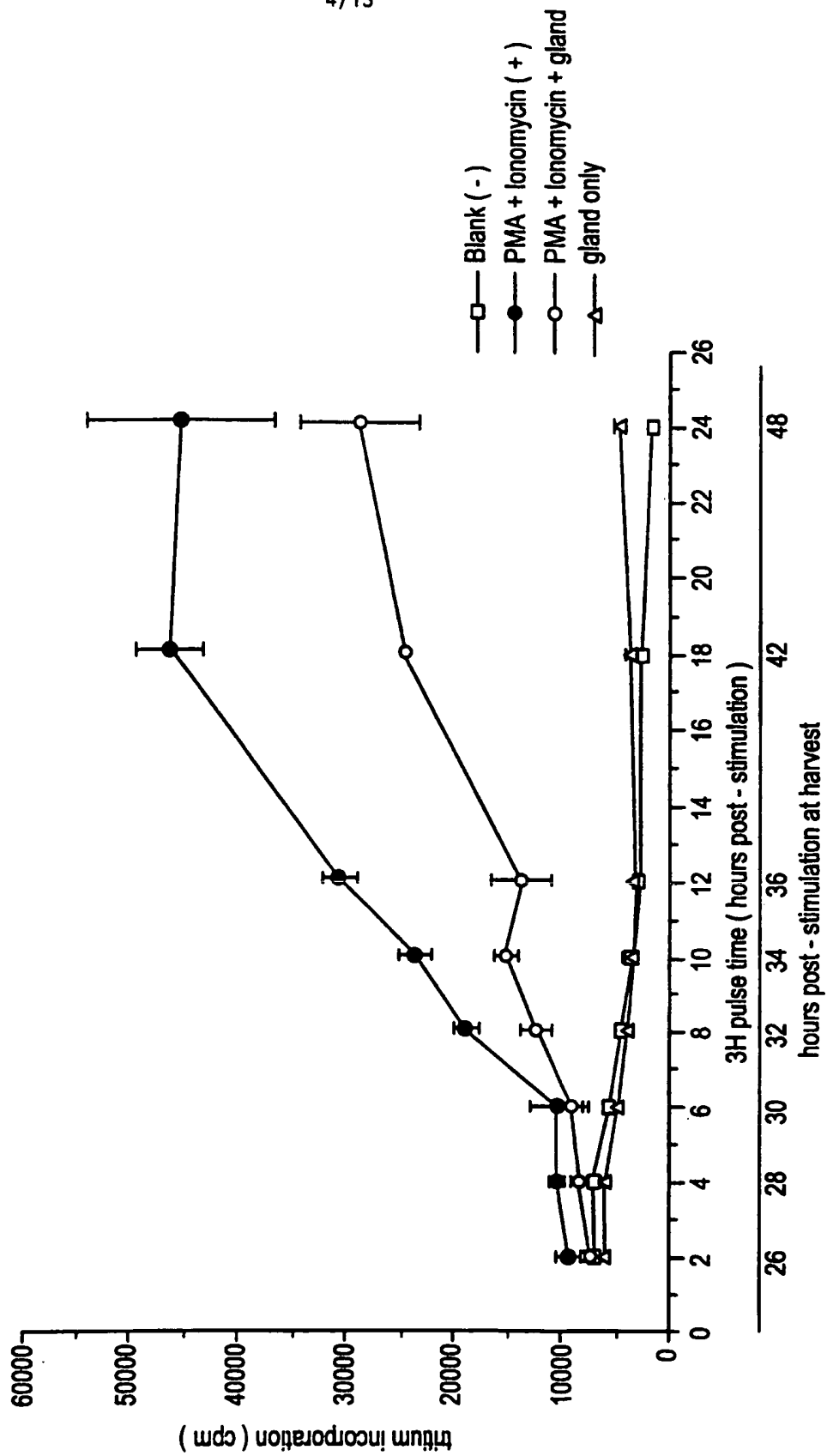


FIG.3



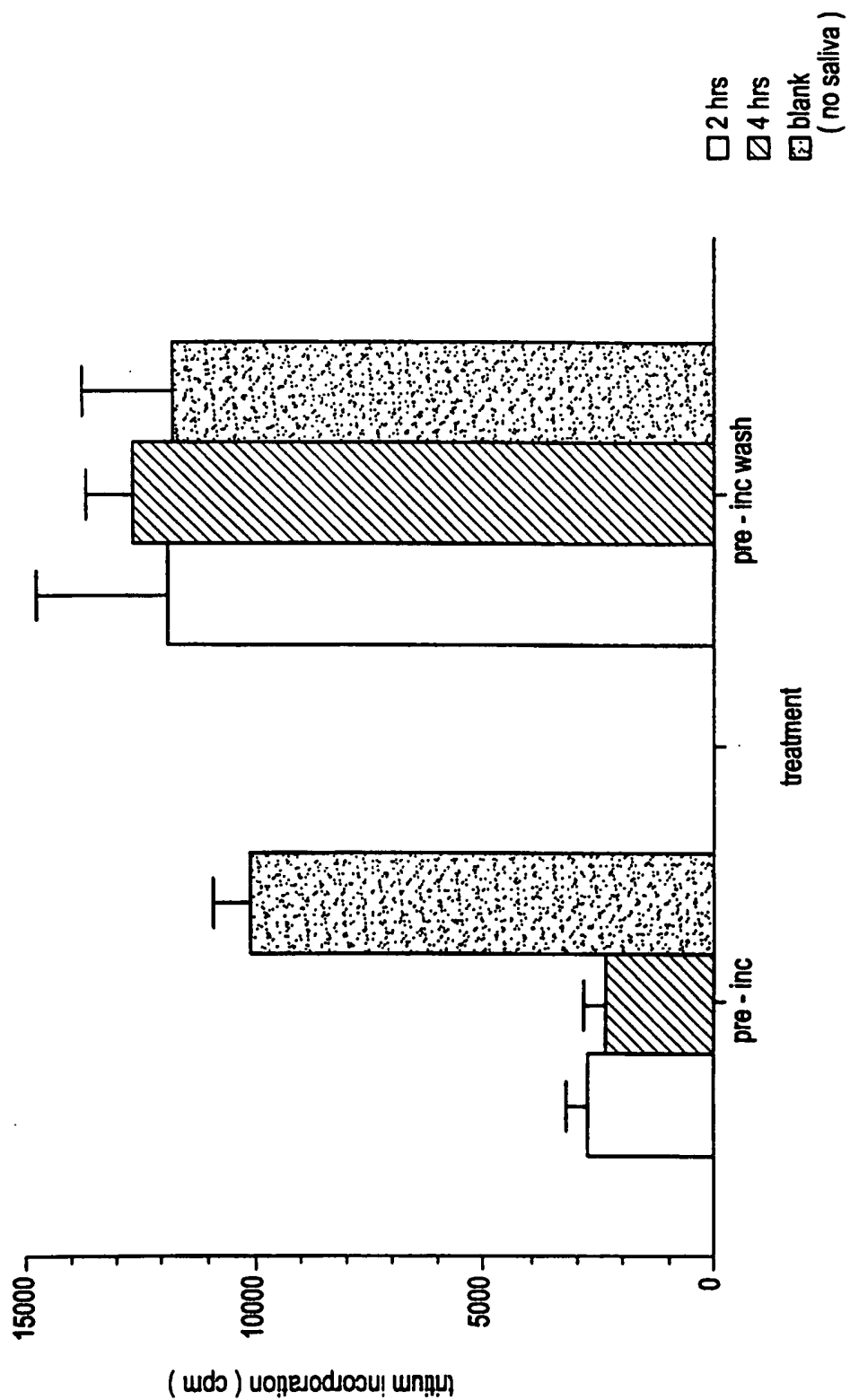
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FIG.4



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FIG.5



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FIG.6A

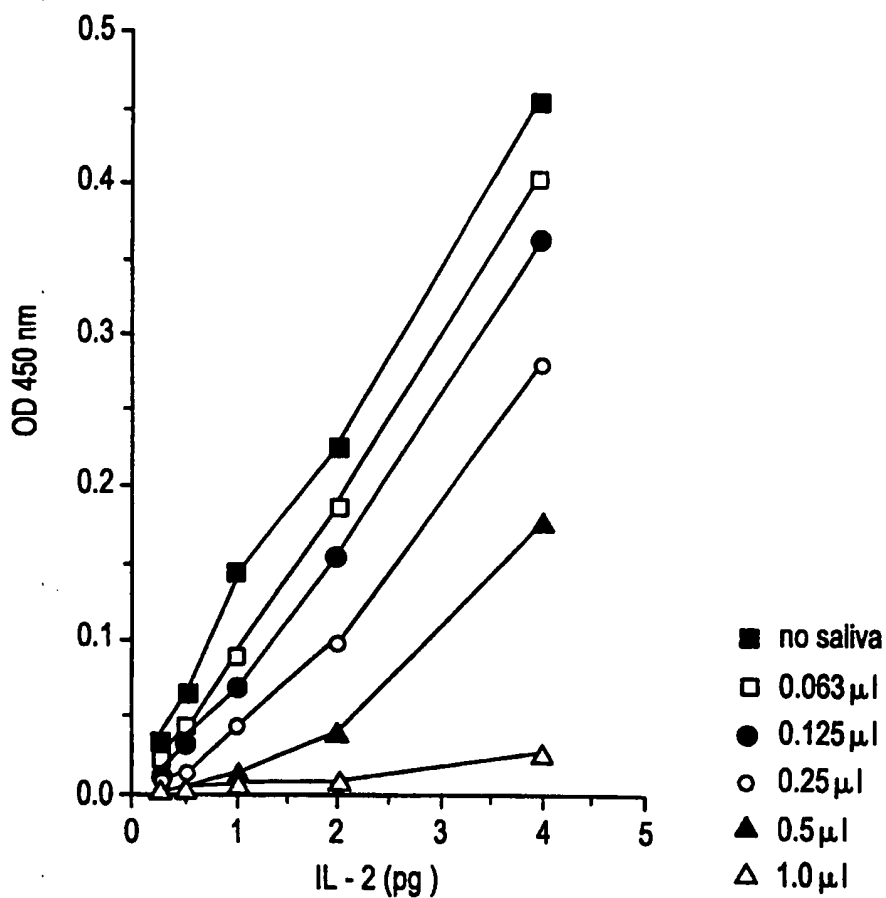
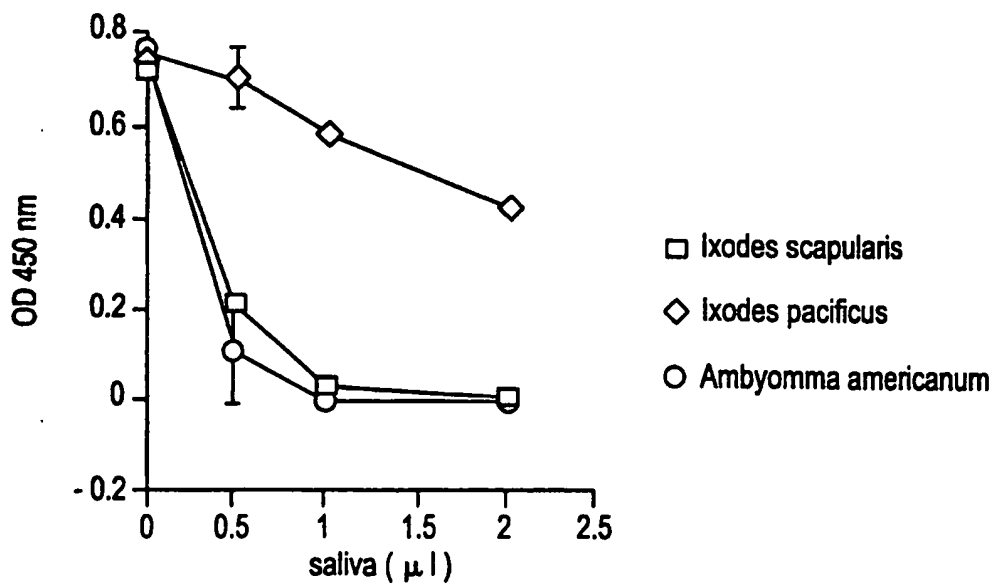


FIG.6B



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FIG.7

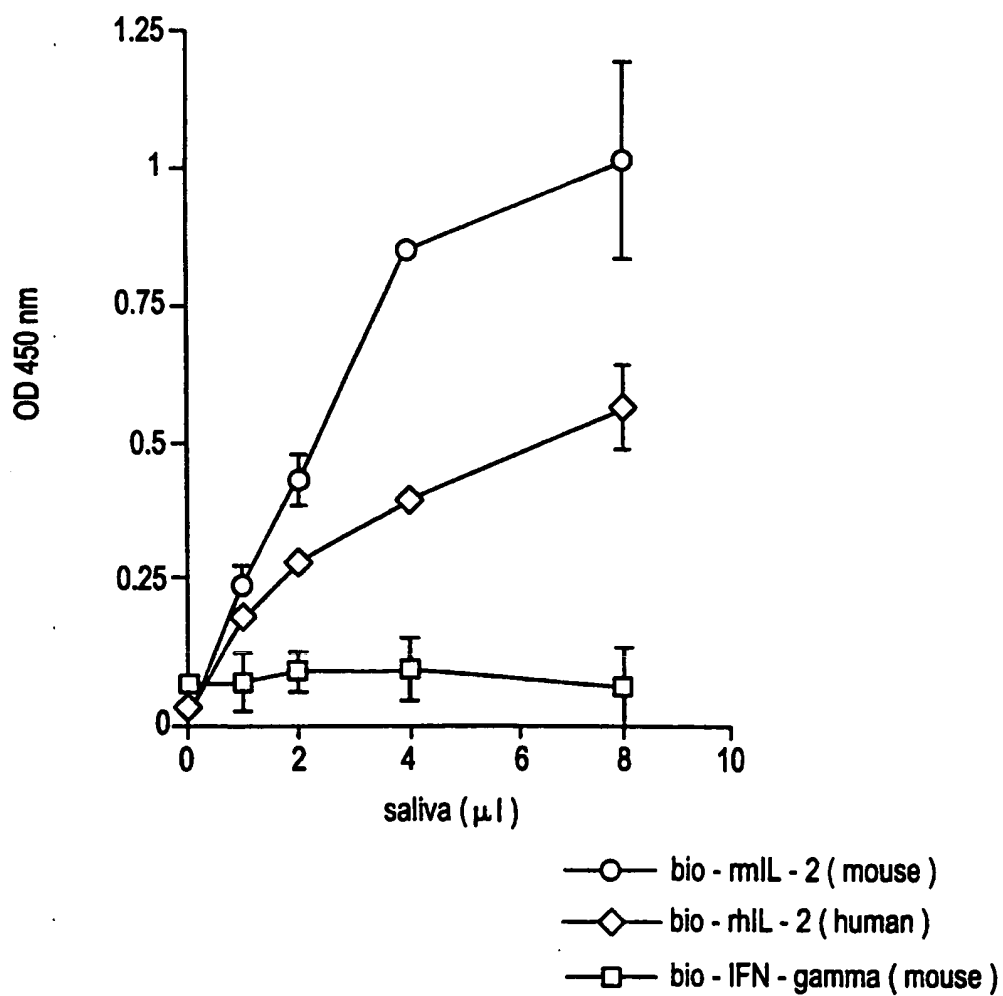
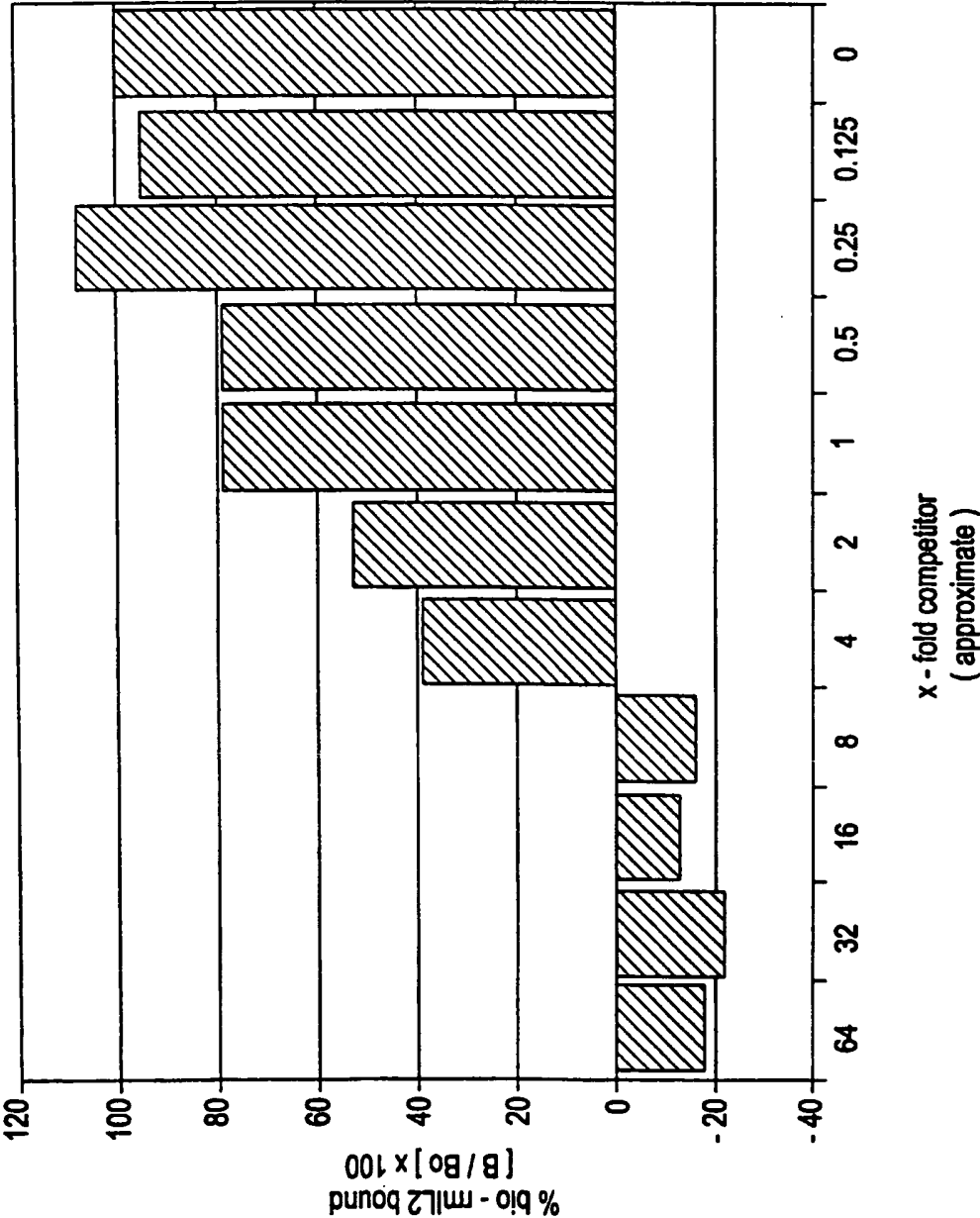
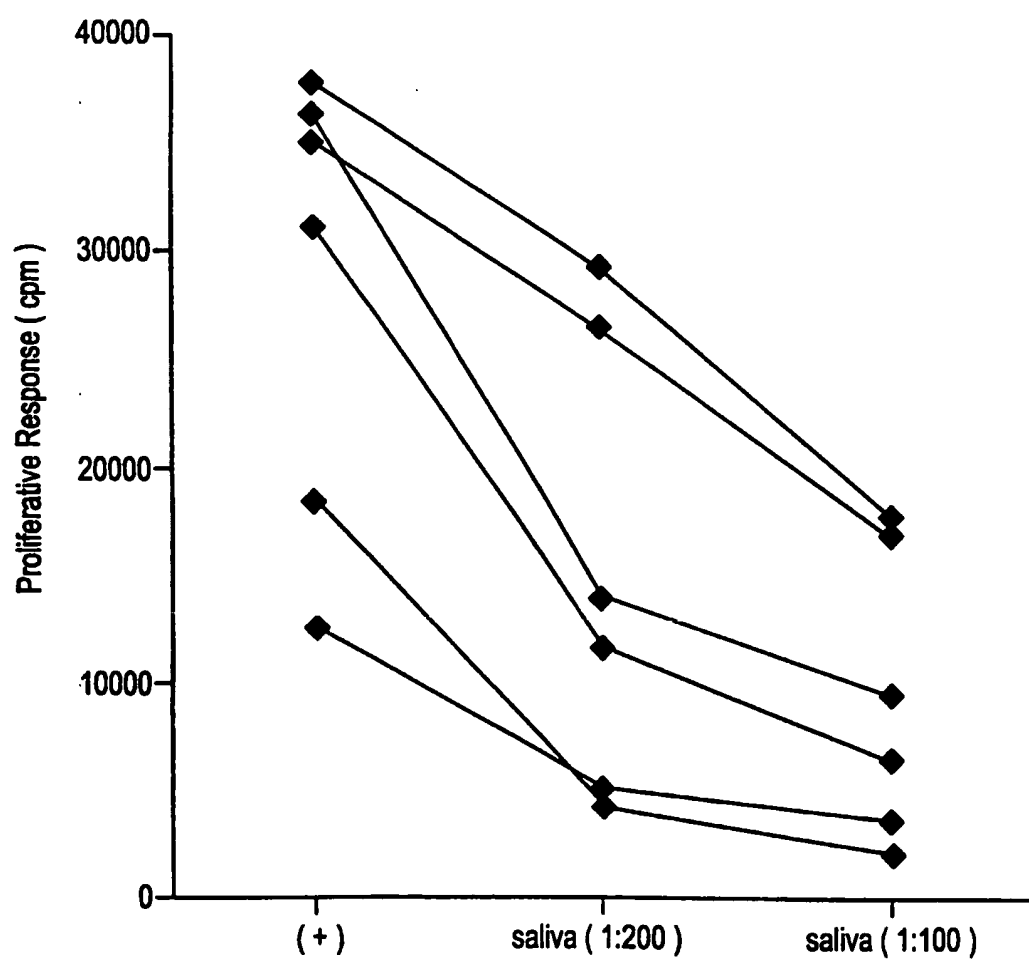


FIG.8



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FIG.9



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FIG. 10C

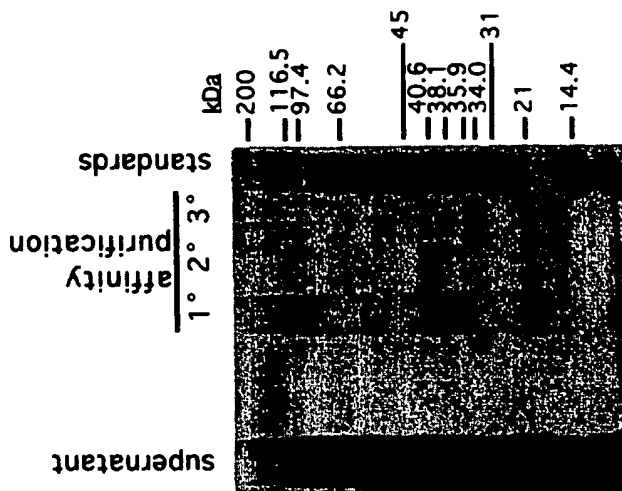


FIG. 10B

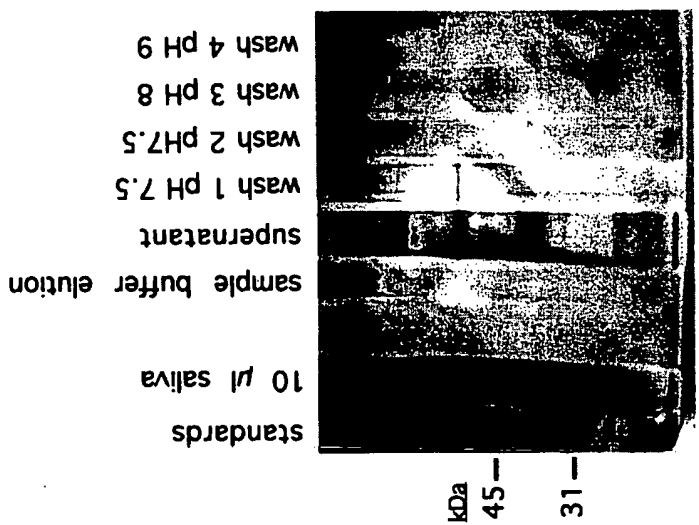
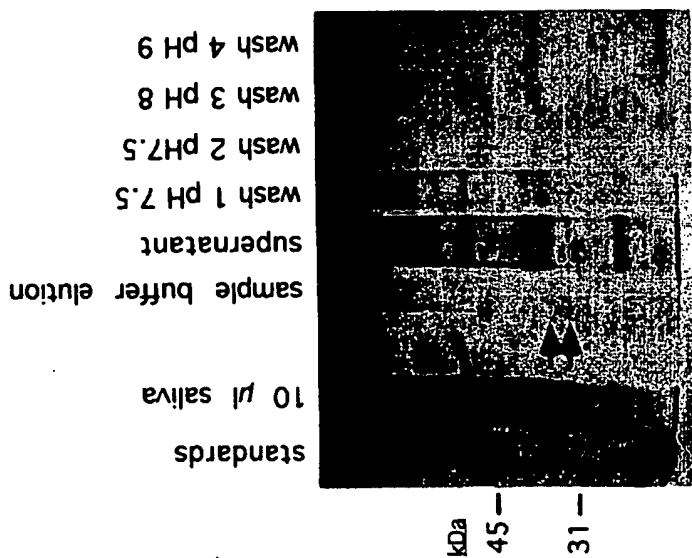


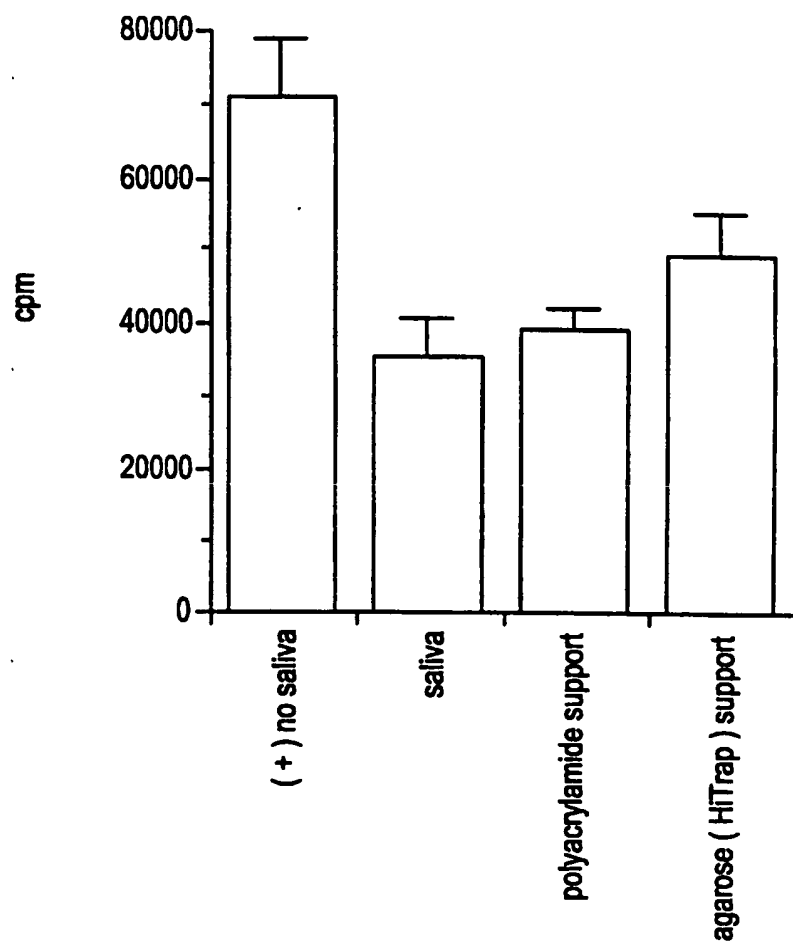
FIG. 10A



↑ calculated band sizes: 35.8 and 32.6

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FIG.10D



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FIG. 11A

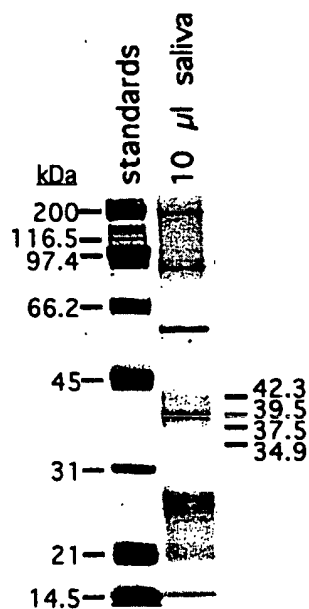


FIG. 11B

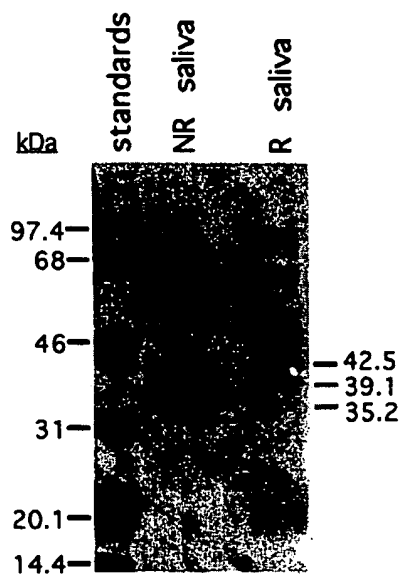
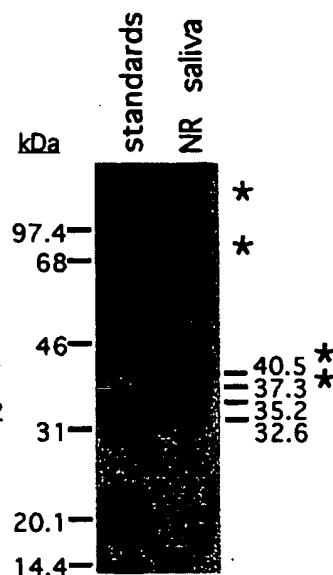
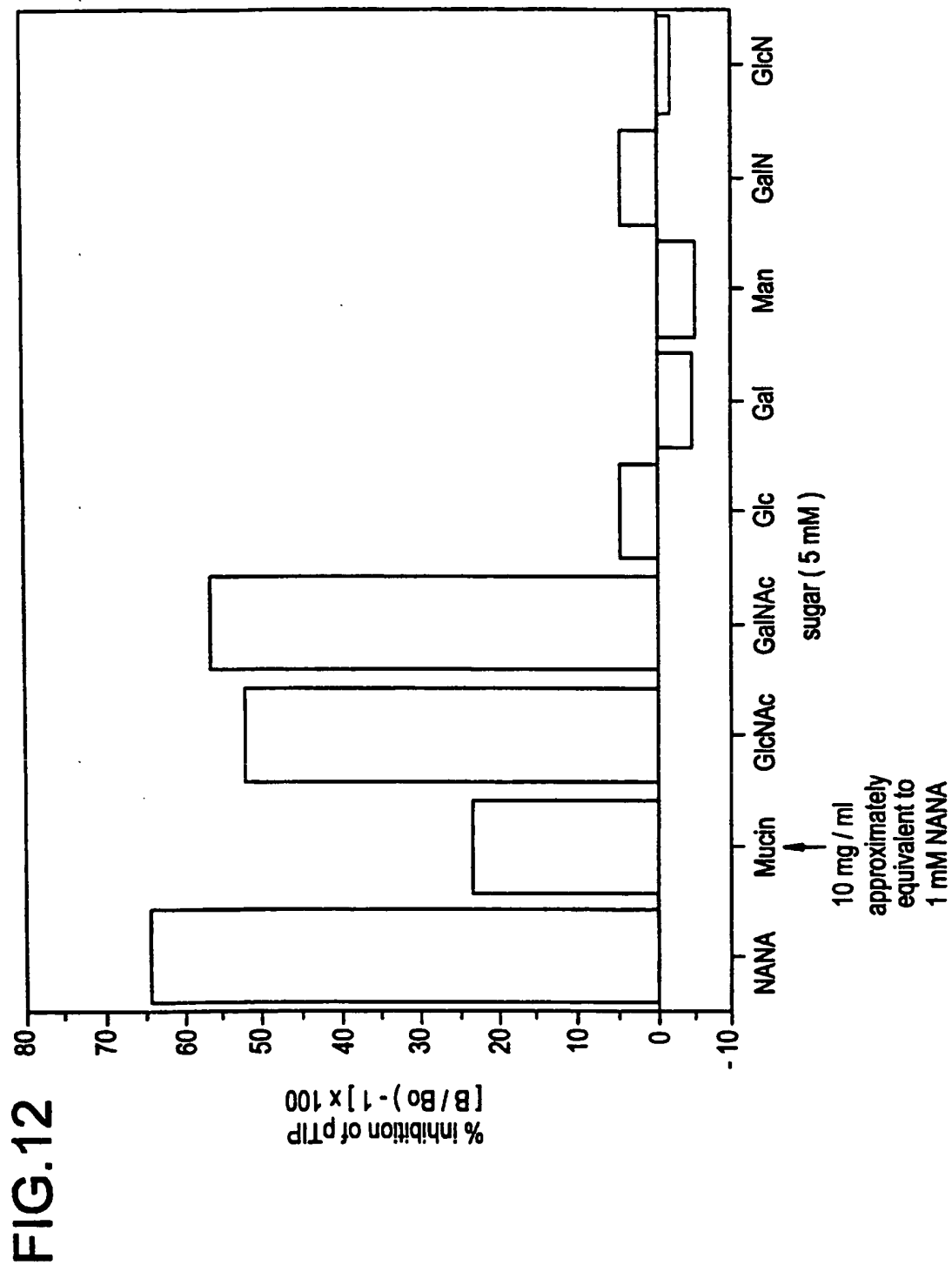


FIG. 11C



bands are the same size

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## INTERNATIONAL SEARCH REPORT

International Application No

PC1, JS 99/26197

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/435 C07K16/18 A61K38/17 A61K39/395 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERGMAN, D.K., ET AL.: "Characterization of an Immunosuppressant Protein from Dermacentor andersoni (Acari: Ixodidae) Salivary Glands" JOURNAL OF MEDICAL ENTOMOLOGY, vol. 35, no. 4, July 1998 (1998-07), pages 505-509, XP000881825	1-4
Y	page 508, left-hand column, paragraph 2 -page 509, left-hand column, paragraph 2 ---	5, 6, 13-21
X	BERGMAN, D.K., ET AL.: "Dermacentor andersoni: Salivary Gland Proteins Suppressing T-Lymphocyte Responses to Concanavalin A in vitro" EXPERIMENTAL PARASITOLOGY, vol. 81, 1995, pages 262-271, XP000881684	1-4
Y	page 268, left-hand column, line 4 -page 269, left-hand column, line 26 ---	5, 6, 13-21
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Date of the actual completion of the international search

15 March 2000

Date of mailing of the international search report

17. 05. 00

Name and mailing address of the ISA

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Morawetz, R

## INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 99/26197

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>URIOSTE, SANDY ET AL: "Saliva of the Lyme disease vector, Ixodes dammini, blocks cell activation by a nonprostaglandin E-2-dependent mechanism." JOURNAL OF EXPERIMENTAL MEDICINE, (1994) VOL. 180, NO. 3, PP. 1077-1085. , XP000881637 see summary and page 1080, right-hand column, paragraph 3 -page 1081, left-hand column, paragraph 1 page 1083, right-hand column, paragraph 2 -page 1084, right-hand column, paragraph 1 ---</p>	1-6, 13-21
Y	<p>WO 98 49303 A (DAS SUBRATA ;FIKRIG EROL (US); KANTOR FRED S (US); UNIV YALE (US);) 5 November 1998 (1998-11-05) page 5, line 30 -page 12, line 9; claims; examples ---</p>	1-6, 17-21
Y	<p>WIKEL, S.K., ET AL.: "Tick-induced modulation of the host immune response" INTERNATIONAL JOURNAL FOR PARASITOLOGY, vol. 24, no. 1, 1994, pages 59-66, XP000881654 ---</p>	13-21
A	<p>page 61, left-hand column, paragraph 5 -page 61, right-hand column, paragraph 1 page 62, left-hand column, paragraph 2 -page 63, right-hand column, paragraph 4 ---</p>	1-6
A	<p>EP 0 238 971 A (HOFFMANN LA ROCHE) 30 September 1987 (1987-09-30) the whole document ---</p>	7,8,11, 12
A	<p>EP 0 621 338 A (AJINOMOTO KK) 26 October 1994 (1994-10-26) page 1, line 3 -page 9, line 14 ---</p>	13-16
P,X	<p>TITUS, R.G.; GILLESPIE, R.D.: "Characterization of an IL-2 binding protein from Ixodes scapularis (tick) saliva" FASEB JOURNAL, vol. 13, 15 March 1999 (1999-03-15), page A954 XP000881956 abstract 704.53 the whole document -----</p>	1-21

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/26197

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 13-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
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4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT, US 99/26197

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9849303 A	05-11-1998	AU 7258498 A	24-11-1998
EP 0238971 A	30-09-1987	DK 134487 A	18-09-1987
		JP 62269698 A	24-11-1987
EP 0621338 A	26-10-1994	JP 7313188 A	05-12-1995
		US 5582826 A	10-12-1996
		US 5856140 A	05-01-1999

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 7 :</b> C07K 14/435, 16/18, A61K 38/17, 39/395, G01N 33/68	<b>A3</b>	<b>(11) International Publication Number:</b> WO 00/27873 <b>(43) International Publication Date:</b> 18 May 2000 (18.05.00)
<b>(21) International Application Number:</b> PCT/US99/26197 <b>(22) International Filing Date:</b> 5 November 1999 (05.11.99)  <b>(30) Priority Data:</b> 60/107,441 6 November 1998 (06.11.98) US  <b>(71) Applicant:</b> RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; Suite 600, 101 North Wilmot Road, Tucson, AZ 85711-3335 (US).  <b>(72) Inventors:</b> TITUS, Richard, G.; 567 Yuma Court, Fort Collins, CO 80525 (US). GILLESPIE, R., Dean; 2212 Vermont Drive, #L204, Fort Collins, CO 80525 (US).  <b>(74) Agents:</b> DIGIGLIO, Frank, S. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 8 September 2000 (08.09.00)
<b>(54) Title:</b> INTERLEUKIN-2 BINDING PROTEIN FROM ARTHROPODS  <b>(57) Abstract</b>  The present invention provides arthropod interleukin-2 binding proteins, antibodies raised against such proteins and agonists and antagonists of such proteins. Methods of purifying such IL-2 binding proteins are also provided. The present invention further provides pharmaceutical compositions containing such proteins, antibodies, agonists or antagonists, as well as the use of such pharmaceutical compositions in protecting animals, including humans, from arthropod infestation and/or the transmission of infectious organisms by arthropods. The therapeutic compositions of the present invention can also be used to modulate the activity of the immune system of animals, including humans.		

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## INTERNATIONAL SEARCH REPORT

International Application No

PC1, JS 99/26197

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/435 C07K16/18 A61K38/17 A61K39/395 G01N33/68

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	page 508, left-hand column, paragraph 2 -page 509, left-hand column, paragraph 2 ---	5,6, 13-21
X	BERGMAN, D.K., ET AL.: "Dermacentor andersoni: Salivary Gland Proteins Suppressing T-Lymphocyte Responses to Concavalin A in vitro" EXPERIMENTAL PARASITOLOGY, vol. 81, 1995, pages 262-271, XP000881684	1-4
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Date of the actual completion of the international search

15 March 2000

Date of mailing of the international search report

17. 05. 00

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Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Morawetz, R

## INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 99/26197

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>URIOSTE, SANDY ET AL: "Saliva of the Lyme disease vector, Ixodes dammini, blocks cell activation by a nonprostaglandin E-2-dependent mechanism." JOURNAL OF EXPERIMENTAL MEDICINE, (1994) VOL. 180, NO. 3, PP. 1077-1085. , XP000881637 see summary and page 1080, right-hand column, paragraph 3 -page 1081, left-hand column, paragraph 1 page 1083, right-hand column, paragraph 2 -page 1084, right-hand column, paragraph 1 ---</p>	1-6, 13-21
Y	<p>WO 98 49303 A (DAS SUBRATA ;FIKRIG EROL (US); KANTOR FRED S (US); UNIV YALE (US);) 5 November 1998 (1998-11-05) page 5, line 30 -page 12, line 9; claims; examples ---</p>	1-6, 17-21
Y	<p>WIKEL, S.K., ET AL.: "Tick-induced modulation of the host immune response" INTERNATIONAL JOURNAL FOR PARASITOLOGY, vol. 24, no. 1, 1994, pages 59-66, XP000881654 ---</p>	13-21
A	<p>page 61, left-hand column, paragraph 5 -page 61, right-hand column, paragraph 1 page 62, left-hand column, paragraph 2 -page 63, right-hand column, paragraph 4 ---</p>	1-6
A	<p>EP 0 238 971 A (HOFFMANN LA ROCHE) 30 September 1987 (1987-09-30) the whole document ---</p>	7,8,11, 12
A	<p>EP 0 621 338 A (AJINOMOTO KK) 26 October 1994 (1994-10-26) page 1, line 3 -page 9, line 14 ---</p>	13-16
P,X	<p>TITUS, R.G.; GILLESPIE, R.D.: "Characterization of an IL-2 binding protein from Ixodes scapularis (tick saliva)" FASEB JOURNAL, vol. 13, 15 March 1999 (1999-03-15), page A954 XP000881956 abstract 704.53 the whole document -----</p>	1-21

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/26197

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# INTERNATIONAL SEARCH REPORT

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9849303 A	05-11-1998	AU 7258498 A	24-11-1998
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		JP 62269698 A	24-11-1987
EP 0621338 A	26-10-1994	JP 7313188 A	05-12-1995
		US 5582826 A	10-12-1996
		US 5856140 A	05-01-1999